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14. ABSTRACT

This revised project had as its goal the characterization of a novel alternative product of the Her-2/neu/erbB2 protooncogene derived from intron retention. The product of this splicing mechanism, termed herstatin, is a secreted protein comprised of the N terminus of the Her-2 receptor tyrosine kinase and a unique, intron-encoded C terminus that allows binding to the other members of the EGFR/erbB family. This binding down-regulates erbB expression and inhibits EGF family signaling and cell proliferation. During this project, we demonstrated that herstatin also down-regulates the IGF-I receptor (IGF-IR) and modulates IGF signal transduction and action, establishing that herstatin is a novel bifunctional inhibitor of erbB and IGF-IR signaling. We also have shown that herstatin is expressed in prostate tissue and prostate cancers of various stages, and can inhibit CaP cell proliferation. We have also demonstrated differential effects of plasmid vs lentivirus-expressed herstatin that will be of relevance to the development of herstatin as a potential therapeutic approach for CaP.

15. SUBJECT TERMS

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Introduction

This revised project focused on the action of an alternative product of the HER-2/erbB2 gene, termed herstatin. Previous work demonstrated that this protein exhibits binding to all members of the erbB family and induces down-regulation of EGFR/erbB1, HER-3/erbB3, and HER-4/erbB4. Herstatin also binds to the IGF-IR. The studies in the revised SOW addressed the regulation of IGF-IR action, its effect on prostate cancer cells in vitro, and its expression in clinical samples from prostate cancer patients. The results of these studies were described in the previous annual reports, including the one for the last year of full funding. We summarize these studies below and also describe additional data obtained in the no-cost extension period ending 12/08. Data from previous annual reports explicitly pertinent to prostate cancer are included in order for this final report to comprise an accurate summary of our prostate cancer-related studies. In summary, this final report represents the work performed during two years of funding following approval of the revised statement of work and one year of a no-cost extension.

Body

In conjunction with the submission of the revised annual report for calendar year 2005, we proposed a modified Statement of Work to guide the research for the remainder of the project. The approved revised SOW comprised the three tasks outlined below.

Task 1. Characterize the effects of herstatin in prostate cancer cells.

Task 2. Evaluate expression of herstatin in prostate cancer cell lines and clinical samples.

Task 3. Investigate regulation of herstatin expression.

Data supporting the revised SOW.

In our annual report following year 1 of this award, we described the effects of constitutive herstatin expression in multiple clones of MCF-7 cells on the expression of various components of the IGF signaling system and IGF-I receptor signal transduction. These included alterations in the expression levels of the IGF-I receptor, IRS-1, IRS-2, Akt, Erk, and Shc, and a significant decrease in IGF-I-stimulated signaling. These results prompted us to request approval of a revised statement of work to focus on herstatin action, which was subsequently approved. It is important to note that we were required to repeat the extensive series of experiments described in the year 1 annual report when we discovered that the herstatin-expressing MCF-7 cell lines we had obtained from our collaborator Dr. Gail Clinton's laboratory were contaminated with mycoplasma as assessed by PCR. Elimination of mycoplasma and verification of our extensive IGF signaling data consumed a significant portion of the work performed during the first year under the revised statement of work (i.e., year 2 of the this Idea Development Award). Fortunately, almost all of the original results were replicated, with the exception of some of the originally reported proliferation and IRS-2 activation data. These subsequent data, along with the effects of herstatin on basal and IGF-I-regulated apoptosis and PARP cleavage shown below, are detailed in the appended revised manuscript that is being submitted to Oncogene. In parallel studies, we demonstrated (and reported in the year 2 annual report) that herstatin expression enhanced insulin receptor expression and signaling, particularly through the Erk pathway. These data are currently being written up for publication. As also described in a year 2 annual report, we performed an in silico analysis of the herstatin 3'-untranslated region and the corresponding genomic sequence and determined that primate (human and monkey) but not rodent HER-2 intron 9 sequences contain polyadenylation motifs that would allow the generation of a functional herstatin mRNA. These data show that both the ability of intron 8 to encode the herstatin protein and the adjacent intron sequences to support mRNA processing are unique to primate species.

In the recent no-cost extension period, we have demonstrated that herstatin expression in MCF-7 cells increases basal apoptosis in serum-starved cells as assessed by PARP cleavage (Figure 1 on the following

page) as well as IGF-I inhibition of apoptosis as assessed by ELISA (i.e., 70% vs 30% decrease in apoptosis by 5 nM IGF-I; Figure 2).

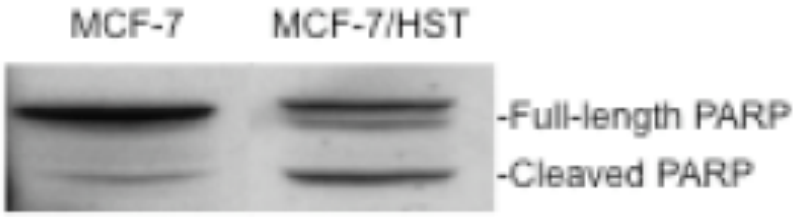

Figure 1. Herstatin expression increases basal PARP cleavage.

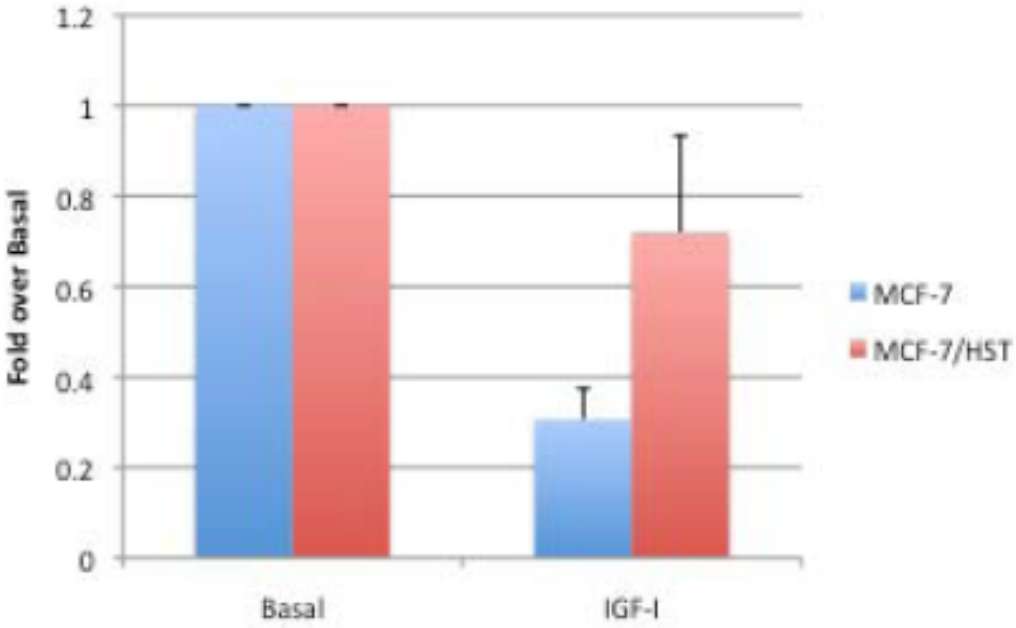

Figure 2. Herstatin inhibits IGF-I attenuation of serum starvation-induced apoptosis.

Progress in direct support of task 1.

We designed and constructed a lentivirus vector encoding herstatin and generated active virus preparations. We infected MCF-7 cells with this preparation to produce cells that acutely express herstatin endogenously. We chose to do these initial infections in MCF-7 rather than prostate cell lines, since the original observations of herstatin effects were made in stably transfected MCF-7 cells. As reported in the year 3 annual report, herstatin-encoding lentivirus-infected cells expressed easily detectable levels of herstatin in total cell lysates using a commercially available herstatin antibody that was raised against the unique intron 8-encoded C terminus of herstatin. No detectable levels of herstatin were detected in control, GFP lentivirus-infected cells. The levels seen in herstatin lentivirus-infected cells were similar to those present in MCF-7 cells stably transfected with a plasmid-based vector described in a previous annual report. In contrast to the effects noted previously in plasmid-transfected cells, acute lentivirus-mediated expression of herstatin had no significant effect on the expression levels of HER-3 or IGF-IR. Thus, the effects of long-term, plasmid-based herstatin expression differ from acute, lentivirus-based expression of similar levels of herstatin. In the no-cost extension period, we found that lentivirus-expressed herstatin is retained intracellularly, with none detected in conditioned media. These data are similar to those reported by Hu et al. (1), who reported intracellular accumulation of herstatin expressed in CHO cells. This may explain the relative lack of effect of lenti-virus-encoded herstatin on expression of the receptor tyrosine kinases that are down-regulated in cells stably transfected with herstatin-encoding plasmid vectors.

The apparent lack of efficient secretion of herstatin expressed from constructs employing the endogenous Her-2 signal peptide may be due to the inability of signal peptides found in type-I transmembrane proteins such as Her-2 to support extracellular secretion in addition to entry into the secretory pathway per se. This notion is supported by the work of Barash et al. (2), who employed hidden Markov modeling to analyze a large collection of mammalian signal peptide sequences and their ability to support secretion of a heterologous protein (alkaline phosphatase). Their analysis found that signal peptides associated with completely secreted proteins exhibited high scores in their model, while those for type-I transmembrane proteins had low scores. Their hidden Markov model was then used to generate a synthetic signal peptide sequence with a very high score that supported efficient secretion of alkaline phosphatase. In the no-cost exrtension period, we designed a herstatin expression vector with this signal sequence (MWWRLWWLLLLLLLLWPMVWA) to enable efficient secretion of herstatin in Cos cells. We will employ this strategy to produce soluble extracellular herstatin for purification as well as for assessing its effect on prostate cancer cells in planned co-culture experiments in transwell plates. These studies will be supported by new funding obtained from OHSU based upon the progress made possible through this PCRP grant. The application for this funded project that will continue development of herstatin as a cancer therapy is included as an appendix.

In the no-cost extension period, we also showned that recombinant herstatin originally produced by Dr. Gail Clinton's laboratory at OHSU is able to inhibit the proliferation of human breast (BT474), prostate (DU145), and stomach (NCIN87) cancer cells as assessed by MTS assay (Figure 3).

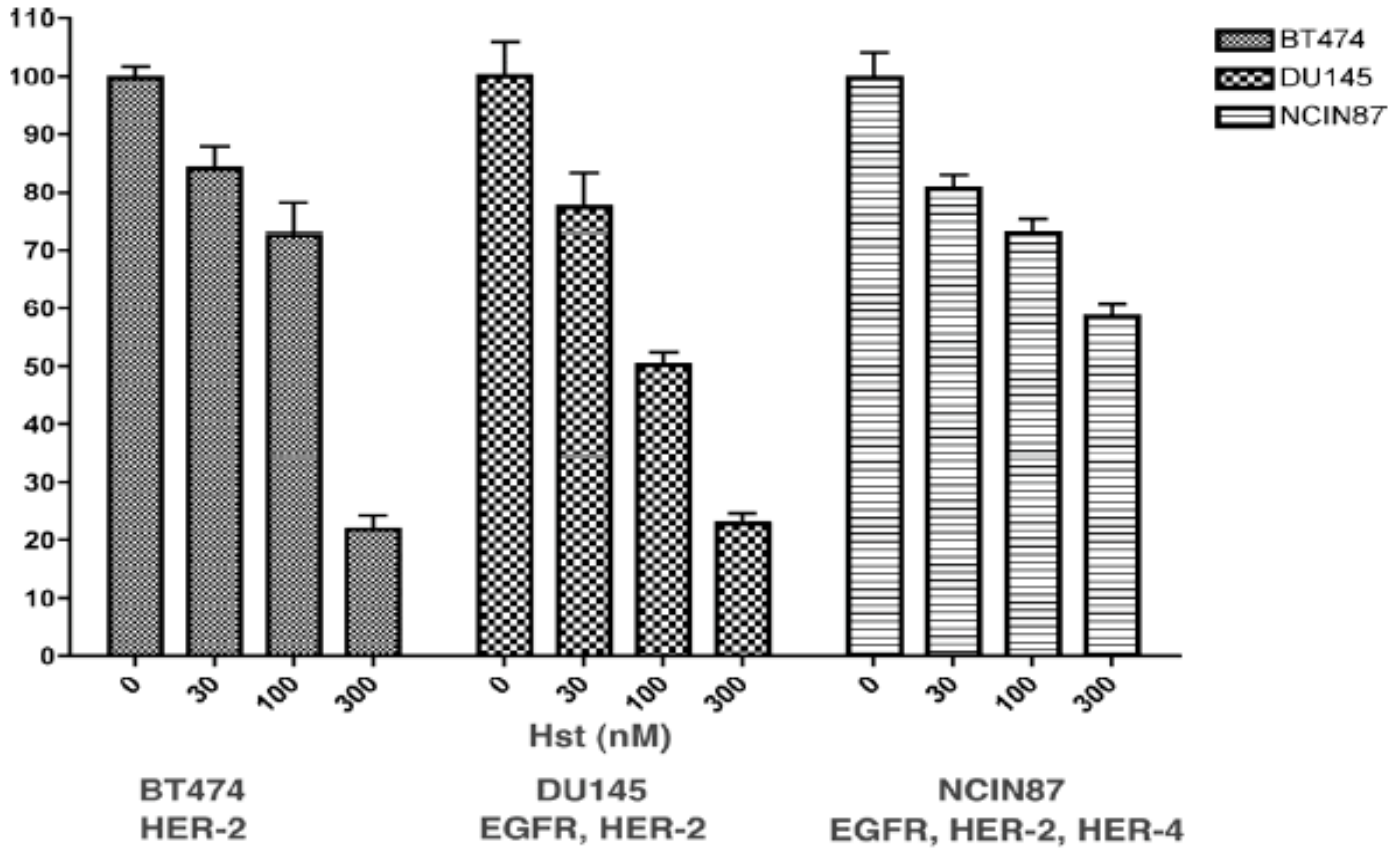

Figure 3. Effect of recombinant herstatin on growth/survival of human cancer cell lines.

We have also demonstrated that recombinant herstatin effectively inhibited EGF-stimulated Akt activation in DU145 prostate cancer cells (Figure 4 on the following page). These studies consumed the entire stock of active recombinant protein previously generated in Dr. Clinton's laboratory. The retirement of Dr. Clinton and the closing of her laboratory and the production of recombinant herstatin in an insect system several years ago

is the rationale for the development of a new system for production of recombinant protein in a mammalian cell system as described above.

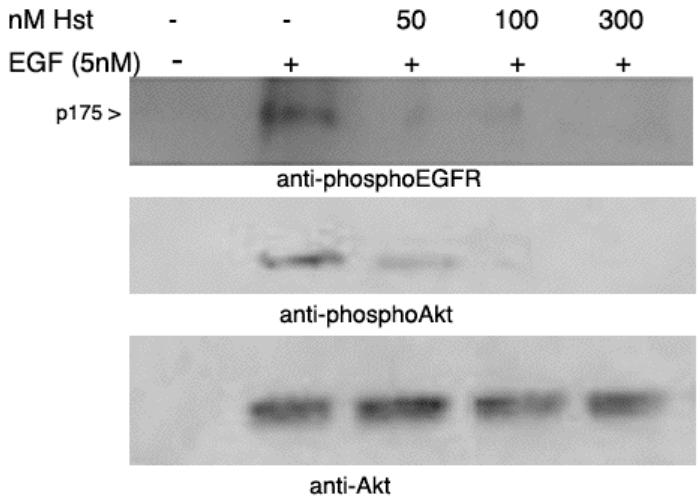

Figure 4. Recombinant herstatin inhibits EGF activation of Akt/PKB in DU145 prostate cancer cells.

Progress in direct support of task 2.

We proposed to evaluate endogenous herstatin expression in various prostate cancer cell lines and a limited number of clinical samples available from the OHSU Cancer Center tumor bank. As shown below in Figure 5, we analyzed endogenous herstatin protein expression in primary prostate epithelial cells and a series of prostate cancer cell lines by western immunoblotting. MCF-7/Hst cell lysate was used as a positive control. Of the prostate cell lines analyzed, DU145 and PC-3 cells appeared to express detectable levels of endogenous herstatin, although these levels were much less than the levels present in MCF-7 cells expressing recombinant herstatin.

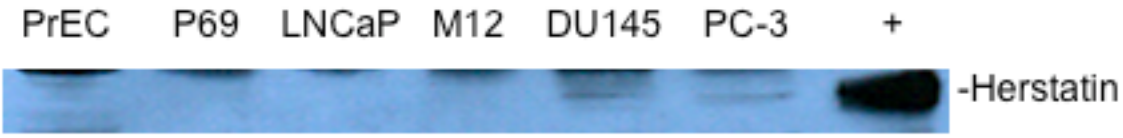

Figure 5. Endogenous herstatin expression in prostate cancer cell lines.

We also investigated herstatin gene expression in deidentified clinical samples comprised of matched normal prostate and adjacent prostate cancer from the OHSU tumor bank by RT-PCR using primers in Her-2 exon 7 and intron 8 that are specific for the herstatin mRNA. This primer set produces a 357-nt product, was comprised of the following sequences, exon 7 FWD 5'-CACTTCAACCACAGTGGCAT-3' and intron 8 REV 5'-GTCCCAAGAGGGTCTGAGGA-3'), and was validated with herstatin cDNA and MCF-7/Hst cell RNA controls. As shown in Figure 6 on the following page, herstatin transcripts were detectable in all normal and matched tumor samples. The presence of herstatin mRNA in all of the normal prostate samples is consistent with the detection of herstatin mRNA in normal prostate using Northern analysis of a multiple tissue blot as described in our year 1 annual report.

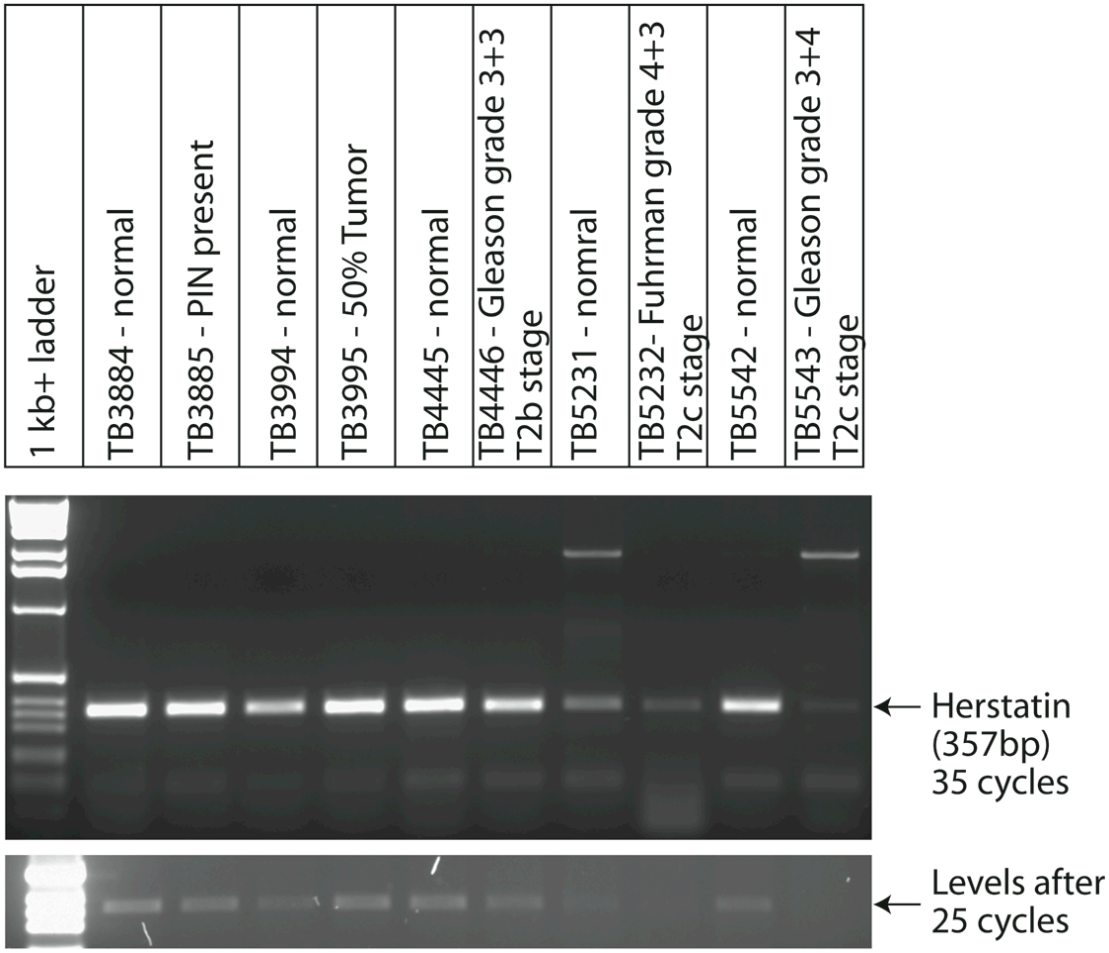

Figure 6. Endogenous herstatin gene expression in matched normal and tumor samples from prostate cancer patients.

In light of potential concerns about the relevance of commonly used prostate cancer cell lines and the relatively small number of well characterized clinical samples available from the OHSU tumor bank, we exploited the commercial availability of an RNA array containing normalized total RNA samples from normal prostates, different prostate cancer stages, and BPH. As described in the previous annual report, this array was analyzed by qRT-PCR (TaqMan technology) using a herstatin-specific amplicon consisting of primers in Her-2 exon 7 and intron 8 that produced an 87-nt product and an internal reporter primer. Primer sequences were: Exon 7 FWD 5'-GGACCTAGTCTCTGCCTTCTACTCTCT-3'; Intron 8 REV 5'-CCCCTCCCCACACTGACA-3', and reporter %'-FAM-CTGGCCCCCCTCAG-MGBNFQ-3'. In control experiments, this probe gave a robust signal with RNA from MCF-7 cells transfected with a herstatin expression vector, but not with control cells. As shown in Figure 7 (and in the previous annual report), we found that endogenous herstatin mRNA levels were similar in an extensive set of normal, prostate cancer, and BPH samples, although there was some individual variability and some samples with particularly high relative expression. The average expression level (Figure 8) was significantly higher in stage II and II prostate cancer than in normal prostate, while the apparently higher level in BPH was influenced by one high outlier. Thus, while herstatin mRNA is present in normal prostate, its expression level was not decreased in prostate cancer as we would have predicted, but, rather, was somewhat increased. This trend of increased expression in prostate cancer may possibly reflect a compensatory response in prostate cancer cells to prevent transformation. These data are similar to recently published findings in breast cancer, where herstatin expression was elevated in breast tumors vs normal tissue (3).

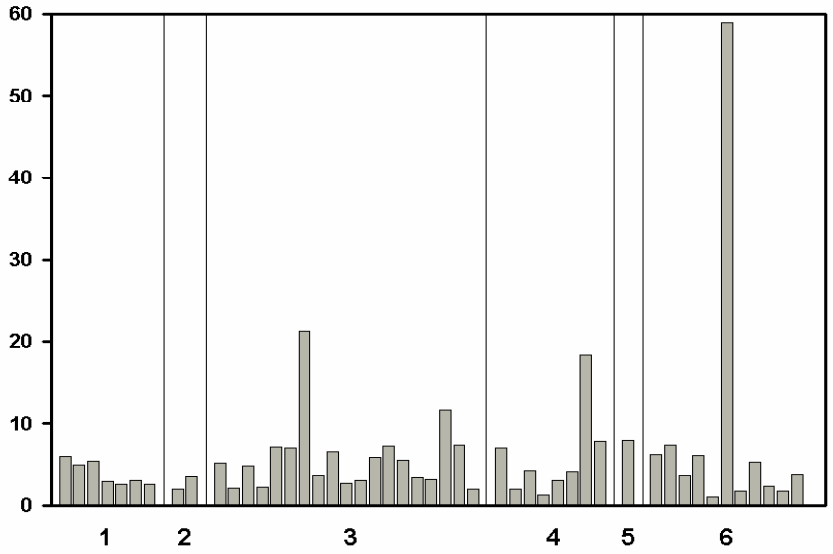

Figure 7. Herstatin mRNA levels in normal prostate, prostate cancer, bladder cancer, and BPH samples by qRT-PCR.

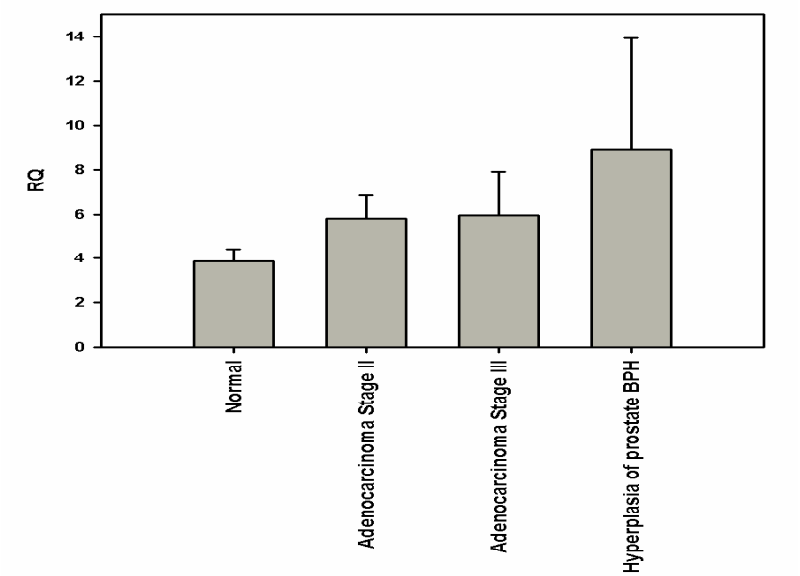

FIGURE 8. Average herstatin mRNA levels in normal prostate, prostate cancer, and BPH samples by qRT-PCR.

Progress in support of task 3.

With the minimal carryover of funds for the no-cost extension period, we were unable to perform experiments pertinent to task 3 in addition to the other data obtained. These studies will be pursued if additional new funding is obtained.

Key research accomplishments

- Demonstration of herstatin regulation of IGF system components and signaling
- Demonstration of herstatin effects on basal and IGF-I-inhibited apoptosis

- Demonstration of herstatin effects on insulin signaling and action
- Characterization of genomic basis for primate-restricted herstatin transcription
- Lentiviral expression of herstatin
- Demonstration of differential effects of acute lentivirus vs chronic plasmid-based herstatin expression on presumptive targets
- Demonstration of differential secretion of herstatin expressed as the endogenous prepeptide
- Demonstration of endogenous herstatin expression at RNA and protein levels in a series of prostate cancer cell lines and in clinical prostate cancer samples and normal prostate controls
- Demonstration of inhibition of prostate cancer cell proliferation by recombinant herstatin
- Demonstration of inhibition of EGFR signaling in prostate cancer cells by recombinant herstatin

Reportable outcomes

- Manuscript for submission to Oncogene acknowledging PCRP support (appended)
- Proposal for additional funding submitted to CLF Medical Technologies Acceleration Program, Inc., through Oregon Health and Science University
- Proposal to Oregon Health and Science University BioScience Innovation Program funded April 2009 for development of herstatin as a cancer therapeutic agent (appended)
- Provisional patent file through Oregon Health and Science University on herstatin modulation of IGF action as a cancer therapy
- Provisional patent filed through Oregon Health and Science University on use of herstatin as a potential insulin sensitizer

Personnel supported by this award

Charles Roberts, Ph.D. (PI); Julie Carroll, M.S. (Research Associate); Elaine Hart, B.S. (Research Assistant).

Conclusions

Our studies supported by this Idea Development Award have established herstatin as a potential therapeutic agent for prostate cancer in addition to other cancers, including breast, ovarian, and stomach cancer, and have demonstrated a novel mode of action that includes simultaneous targeting of both the EGFR/HER/erbB and IGF-IR families. This activity profile supports the potential superiority of herstatin-based therapeutics over current monoclonal antibody and small-molecule inhibitors that lack this spectrum of effects. The potential clinical and commercial possibilities for therapeutic development of herstatin have been validated by our recent success in procuring follow-up funding support that will enable the furtherance of the research initiated through this Award.

References

1. Hu P. et al. Sequestering ErbB2 in endoplasmic reticulum by its autoinhibitor from translocation to the cell surface: an autoinhibition mechanism of ErbB2 expression. Biochem. Biophys. Res. Comm. 342:19-27 (2005).

2. Barash S., Wang W., Shi Y. Human secretory signal peptide description by hidden Markov model and generation of a strong artificial signal peptide for secreted protein expression. Biochem. Biophys. Res. Comm. 294:835-842 (2002).

3. Koletsa T. et al. A splice variant of HER2 corresponding to herstatin is expressed in the noncancerous breast and in breast carcinomas. Neoplasia 10:687-696 (2008).

Appendices

1. Revised Oncogene manuscript citing PCRP support

2. Funded OHSU BioScience Innovation Program project for further development of herstatin as a clinical therapeutic, including additional studies in prostate cancer models

Supporting data.

NA

MODULATION OF INSULIN-LIKE GROWTH FACTOR SIGNALING BY HERSTATIN, AN ALTERNATIVELY SPLICED HER-2 (erbB-2) GENE PRODUCT

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Running title: Herstatin regulation of IGF signaling

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ABSTRACT

Herstatin, a product of alternative splicing of the HER-2 gene, is comprised of subdomains I (L1) and II (S1) of the HER-2 receptor tyrosine kinase, followed by a 79-amino acid C-terminal domain encoded by intron 8. We have previously shown that herstatin binds to multiple members of the EGF receptor (EGFR/erbB/HER) family, and that binding to EGFR and HER-2 blocks dimerization and ligand activation. Herstatin also binds to the IGF-I receptor (IGF-IR), which exhibits signaling crosstalk, and contains regions of high homology with, the EGFR family. We have extended these findings by investigating the effects of herstatin on IGF system expression and action in MCF-7 breast cancer cells. IGF-IR expression and IGF-stimulated IGF-IR tyrosine phosphorylation were significantly reduced in two different clones of herstatin-expressing cells. These effects were not caused by herstatin-mediated inhibition of the EGFR, since treatment of parental MCF-7 cells with an EGFR-specific inhibitor, AG1478, for up to 24 hours did not affect IGF-IR levels. Herstatin also inhibited the expression and IGF-induced tyrosine phosphorylation of IRS-1, while IRS-2 expression and activation was not affected. Although IGF-IR and IRS-1 tyrosine phosphorylation was strongly reduced, herstatin did not inhibit, but stimulated, IGF-I-mediated ERK activation, and IGF-I activation of the PI3K-Akt/PKB pathway was modestly inhibited. Herstatin also binds to the insulin receptor, and constitutive herstatin expression up-regulates insulin receptor expression and activation. These studies demonstrate that herstatin inhibits both erbB/HER and IGF-IR signaling while enhancing insulin receptor expression and action. Together, our data establish herstatin as a broad-spectrum inhibitor of EGF family and IGF action with potential therapeutic potential in prostate, breast, and other cancers in which both erbB/HER and IGF-IR have been implicated.

INTRODUCTION

Receptor tyrosine kinases (RTKs), including the epidermal growth factor receptor (EGFR) and the insulin-like growth factor-I receptor (IGF-IR) families, play critical roles in fundamental cellular processes. The EGFR family, which includes the EGFR (HER-1/erbB1), HER 2/neu/erbB2, HER-3/erbB3, and HER 4/erbB4, has been shown to mediate key cellular processes such as growth and differentiation (2-4). The IGF-IR family, which includes the IGF-IR, the insulin receptor, and the insulin receptor-related receptor, has also been shown to participate in an overlapping array of biological processes (5-11). While the expression and biological effects of these receptor families are essential for normal growth and development, aberrant expression leads to a variety of human cancers (12-15).

The four members of the EGFR family each contain an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain (16-18). Eleven ligands, each containing an EGF core domain, bind with high affinity to these receptors, except HER-2, causing the formation of receptor homo- or heterodimers. This dimerization results in receptor activation and autophosphorylation of specific C-terminal tyrosine residues (4,17,19-22), which enables the subsequent recruitment and tyrosine phosphorylation of SH2-domain-containing signaling molecules, leading to the initiation of two major intracellular signaling pathways, the (generally) anti-apoptotic PI3KAkt/PKB and mitogenic ERK cascades (12,23,24).

The IGF-IR, in contrast to most other RTKs, consists of a pre-formed, disulfide-linked, heterotetramer (25,26). Ligand binding to the extracellular α subunits leads to a conformational change in the transmembrane β subunits and autophosphorylation of tyrosine residues in the catalytic domain. The subsequent phosphorylation of additional tyrosines, particularly in the juxtamembrane domain of the β subunit, provides docking sites for PTB and SH2-domain-containing scaffolding/adapter proteins, including the insulin receptor substrates IRS-1 and IRS-2. These adaptor proteins then activate signaling pathways such as the PI3K and ERK cascades that are also activated by the EGFR family (27).

Both the EGFR and IGF-IR families are major regulators of cell growth and survival, and dysregulation of either receptor family can lead to uncontrolled growth and tumorigenesis. Recent evidence suggests that there is crosstalk between these RTKs, which may allow coordinated control of cellular responses in normal and tumor cells

(reviewed in (1)). Sustained activation of a mitogenic ERK signal by the EGFR is dependent on a functional IGF-IR (28). Recently, the converse was also shown to be true, in that activation of ERK by the IGF-IR requires a functional EGFR (5,29,30). Additionally, it has been shown in several cell types that IGF-I stimulation of the IGF-IR leads to activation of the EGFR and, coordinately, the ERK pathway, through proteolytic activation and autocrine release of HB-EGF (30-32). IGF-I-induced coordinate activation of ERK through EGFR and IGF-IR is in contrast to IGF-I-induced activation of Akt, which is unaffected by EGFR-specific inhibitors (30,32). These data suggest that crosstalk between the EGFR and the IGF-IR controls activation of the ERK signaling pathway, but not the PI3K-Akt/PKB pathway. In addition to coordination of signal transduction, Ahmed et al. have recently reported that the EGFR co-immunoprecipitates with the IGF-IR in mammary epithelial cells, and that phosphorylation of the complexed EGFR is enhanced by treatment with IGF-I (29). Another recent study has described an association between IGF-IR and HER-2 (X).

Because of the important role of the EGFR family in malignant growth, extensive effort has been directed toward the development and characterization of specific inhibitors. Effective tumor inhibition has been achieved clinically with inhibitors that antagonize the EGFR and HER-2 (33,34). Several studies suggest that redundant signaling through the IGF-IR maintains the activation of pathways necessary for survival in the presence of EGFR family inhibitors. *In vitro*, IGF-IR signaling in MCF-7/HER-2 and SKBR-3 breast carcinoma cells protects against inhibition by Herceptin, an anti-Her-2 monoclonal antibody (35). The inhibitory effects of AG1478, an EGFR inhibitor, can also be overcome in glioblastoma multiforme cells by overexpression and increased signaling through the IGF-IR (36). Most recently, it has been shown in breast and prostate cancer cell lines that acquired resistance to Iressa, a small-molecule EGFR inhibitor, occurs through increased IGF-IR activation and signaling (37,38).

Recent efforts have also been directed at targeting the IGF-IR family. Inhibition of tumor growth with two IGF-IR small-molecule inhibitors has been documented with solid tumor xenografts and leukemic malignancies (39,40). Specific anti-IGF-IR antibodies have been recently developed that have shown efficacy in inhibition of IGF-stimulated proliferation and tumorigenesis (41-43). Additionally, *in vitro* combinatorial therapy, using Herceptin to block HER-2, and a dominant-negative form of the IGF-IR in breast carcinoma cells, revealed synergy between the two treatments and led to increased growth inhibition (44). Recently, a bivalent monoclonal antibody to the EGFR and IGF-

IR has been described (45,46). Use of this di-antibody resulted in increased growth inhibition compared to that achieved with either anti-EGFR or anti-IGF-IR antibodies alone (45).

In this study, we investigated the impact of a cellular pan-EGFR family inhibitor, herstatin, on IGF-I signaling. Herstatin, the product of alternative splicing of the HER-2 gene transcript, consists of the N-terminal portion of the HER-2 RTK, followed by a novel 79-amino acid C-terminal domain (47). Herstatin is unique in that it binds with nM affinity to all members of the EGFR family (48), and its binding to EGFR and HER-2 blocks receptor activation (47,49-51). We have recently demonstrated that herstatin also binds with lower affinity to the IGF-IR compared to the EGFR (Kd≈150 nM vs 15 nM) (48), presumably to a site in the ectodomain that has homology with the EGFR (52). We, therefore, determined the effects of herstatin on IGF-I signaling system expression and signaling in MCF-7 mammary carcinoma cell lines. Our data demonstrate that herstatin action represents a novel mechanism of cross-regulation of the EGFR and IGF-IR families.

MATERIALS AND METHODS

Cell culture

MCF-7 breast carcinoma cells were obtained from the American Type Culture Collection and maintained at 37^{o}C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% pen/strep (Gibco). Media and supplements were purchased from Gibco BRL-Life Technologies (Grand Island, NY). Herstatin-expressing MCF-7 clones (MCF-7/Hst cells), previously characterized (50), were maintained under the same conditions as parental MCF-7 cells in media supplemented with 0.5 mg/ml G418 sulfate.

Antibodies

All primary antibodies were used at a 1:1000 dilution unless otherwise indicated. Polyclonal antibodies [IGF-IR, IRS-1 (N-terminus) and PARP] and monoclonal antibody PY20 and were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal ERK 1/2 and polyclonal pERK 1/2, Akt/PKB, and IRS-1 antibodies were purchased from Cell Signaling Technologies (Boston, MA). Monoclonal herstatin and polyclonal IRS-2

antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal pAkt/PKB antibody was purchased from BioSource International (Hopkinton, MA).

Western immunoblotting and immunoprecipitation

Cells were grown to ~80% confluency, serum-starved overnight in DMEM, and treated with 14 nM EGF (Sigma) or 5 nM IGF-I (GroPep, Australia) for the times indicated. For Western blots, cells were washed twice with ice-cold PBS and lysed in 1XSDS sample buffer (53) without reducing agent and boiled for 5 min. After clarification by centrifugation at 13,000 rpm for 5 min., supernatant was collected and protein concentration was determined using a detergent-compatible protein assay kit (Bio-Rad; Hercules, CA). Dithiothreitol (100 mM) and bromophenol blue (0.1% (w/v)) were then added and samples were boiled again for 5 min. Twenty-µg aliquots of protein were analyzed by 10% SDS-PAGE and electrotransferred onto nitrocellulose (Amersham Pharmacia Biotech; Piscataway, NJ). Blots were probed with a phospho-specific antibody, stripped in 5x stripping buffer (53) and reprobed with the respective pan antibody. For immunoprecipitation, cells were washed twice with ice-cold PBS, lysed in NP-40 buffer [1% NP-40, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.2% SDS], containing protease inhibitors (Roche Diagnostics; Indianapolis, IN) and 2mM $NaVO_4$. Lysates were cleared and protein concentration was determined as above. For IGF-IR, 1 mg of whole-cell lysate protein was immunoprecipitated with 5 µg of anti-IGF-IR antibody and incubated overnight at 4^oC while rocking. For IRS-1 and IRS-2, 500 µg of whole-cell lysate protein was incubated overnight at 4^oC with 3 µg antibody. 100 µl of protein A-agarose bead slurry (Amersham Pharmacia Biotech) was added for 2 hours rocking at 4^oC. Three washes were performed, and the pellet was boiled in 2x SDS sample buffer (53). The beads were spun down and the supernatant loaded onto a 10% (IGF-IR) or 7% (IRS-1/2) SDS-PAGE and immunoblotted as above. Blots were probed with PY20, stripped, and reprobed with their respective antibodies. Binding of primary antibodies was detected by enhanced chemiluminescence (Amersham), and film exposures were quantified using a scanning densitometer (Bio-Rad).

Cellular apoptosis

For determination of apoptosis, cells ($3x10^4$) were plated in 96-well plates and grown for 24 hrs under normal culture conditions, incubated in serum-free DMEM for 16 hours,

and treated with either 5 nM IGF-I (Gro-pep; Adelaide, Australia), 30ng/ml TNF-α, both IGF-I and TNF-α, or an equivalent volume of vehicle for 24 hours. Apoptosis was measured via the Cell Death Detection Elisa Plus (Roche Diagnostics).

For assessment of PARP cleavage, cells were seeded into 6-well plates and grown under normal culture conditions until cells were 70-80% confluent. Cells were then exposed to UV radiation (2400 j/m^2) by placing plates on a UV transilluminator for 2 min, 8 sec (1XUV) or 4 min, 16 sec (2XUV). Attached and floating cells were collected and lysed for Western blots as described above at 24 hrs and 48 hrs post-UV exposure and analyzed for PARP cleavage.

EGFR inhibitor studies

Control MCF-7 cells were serum-starved overnight and treated with the EGFR kinase AG1478 (Sigma) or vehicle (DMSO) for 5 min. prior to the addition of 14 nM EGF or 5 nM IGF-I. After 5 min. of growth factor treatment, cell lysates were prepared and analyzed for ERK and Akt/PKB activation as described above.

RESULTS

Effect of herstatin on the expression of IGF signaling molecules

The studies described below demonstrate the effects of herstatin expression on IGF-I signaling. In preparation for those studies, we first examined the effect of herstatin expression on basal levels of the signaling molecules comprising the IGF signaling system. The expression of herstatin in MCF-7 cells resulted in the down-regulation of several components of the IGF signaling system (Fig. 1). Both IGF-IR and IRS-1 protein levels were decreased 5-fold, while IRS-2 protein levels were modestly up-regulated. There was no apparent difference in the levels of total ERK; however, there was a shift from a preponderance of ERK1 to ERK2, as well as an increase in the apparent size of ERK1. Akt/PKB levels were modestly affected, with an average 2-fold decrease seen in herstatin-expressing cells.

Effect of herstatin on IGF-IR activation

To evaluate the effect of herstatin on activation of the IGF-IR by IGF-I, we examined tyrosine phosphorylation of IGF-IR immunoprecipitated from IGF-I-treated MCF-7 and MCF-7/Hst cells. In MCF-7 cells, IGF-I robustly stimulated IGF-IR tyrosine

phosphorylation, which represents the initial autophosphorylation stage of IGF-IR activation. In MCF-7/Hst cells, however, there was only a small increase in IGF-IR phosphorylation, which corresponds to an approximately 8-fold reduction in activation (Fig. 2). This decreased activation reflects, in part, the decrease in IGF-IR expression consistently seen in herstatin-expressing cells (see Fig. 1), as well as diminished tyrosine phosphorylation (Fig. 2). Reduced IGF-IR expression and activation by IGF-I (and IGF-II) were also observed in a second clonal line of herstatin-expressing cells (data not shown).

IGF-I activation of IRS-1 and IRS-2

To further investigate the effects of herstatin expression on IGF-I-mediated signaling, we examined the activation of IRS-1 and IRS-2, signaling molecules immediately downstream of the IGF-IR. IGF-I-induced phosphorylation of IRS-1 was severely reduced in MCF-7/Hst cells compared to control cells (Fig. 3A & B). This decreased tyrosine phosphorylation of IRS-1 was a result of both decreased expression of IRS-1 (~5-fold; see Figure 1), as well as an apparent 6- fold decrease in the efficiency of IRS-1 immunoprecipitation in herstatin-expressing cells. This reduction in the amount of IRS-1 immunoprecipitated from herstatin-expressing cells was also seen with a second, N-terminally directed IRS-1 antibody (data not shown). Together, the combined effects of decreased IRS-1 expression and immunoprecipitation efficiency resulted in an ~30-fold difference in the amount of IRS-1 in immunoprecipitates from control and herstatin-expressing cells. This was similar to the difference in tyrosine-phosphorylated IRS-1; therefore, the decrease in IRS-1 protein immunoprecipitated from herstatin-expressing cells was equivalent to the decrease in IRS-1-associated phosphotyrosine. Thus, the relative activation of IRS-1 was similar in control and herstatin-expressing cells. In contrast, the levels of activated (tyrosine-phosphorylated) IRS-2 were slightly increased in herstatin-expressing cells, which was proportional to the slight increase in IRS-2 protein seen in herstatin-expressing cells (Fig. 3 C & D & Fig. 1).

IGF-I activation of ERK and PKB

Herstatin has been shown to differentially affect ERK pathway activation. Specifically, herstatin had no effect on EGF or TGF-α-stimulated ERK activation in 3T3 cells over-expressing EGFR (49), but did inhibit heregulin activation of ERK in MCF-7 cells (50) and EGF activation of ERK in U87MG cells (54). Herstatin expression did not inhibit

overall activation of the ERK signaling pathway in IGF-I-treated MCF-7 cells. ERK phosphorylation was rapid and transient, with a maximal response at 5 minutes in parental cells. In herstatin-expressing cells, the timing of the maximal response was the same, but the amplitude of total ERK activation, indicated by enhanced phospho-ERK, was enhanced several-fold (Fig. 4). Interestingly, we observed a specific stimulation of ERK2, while there was no change in the activation of ERK1. Furthermore, we consistently observed an increase in the apparent size of ERK1. This may correspond to the appearance of an ERK1 splice variant, or a post-translational modification (55-57). In contrast, IGF-I activation of the PI3K pathway, as assessed by the overall level of Akt/PKB phosphorylation, was reduced by 2-fold in MCF-7/Hst cells (Fig. 5). This effect is similar to the previously reported inhibition of EGF and heregulin-stimulated Akt/PKB activation in 3T3 and MCF-7 cells (49, 50, 54). Thus, herstatin expression did not reduce, but enhanced, ERK2 signaling, but attenuated the anti-apoptotic Akt/PKB signaling cascade. Similar effects, i.e., enhanced ERK2 activation and decreased Akt/PKB activation, were also seen in a second, independent herstatin-expressing MCF-7 clone (data not shown).

Herstatin increases basal apoptosis and inhibits IGF-I protection from apoptosis

Previous studies have shown that stable expression of herstatin in MCF-7 cells blocked heregulin-stimulated proliferation (50). The inhibition of IGF-IR signaling observed in herstatin-expressing cells suggested that herstatin may also interfere with IGF-I-mediated growth and survival. To further investigate the effect of herstatin on IGF-I action, we assessed the potential effects of herstatin on IGF-I action. Control MCF-7 cells exhibited a modest response to IGF-I in terms of proliferation, making a determination of the effects of herstatin on this parameter difficult. Expression of herstatin reduced IGF-I inhibition of serum-starvation induced apoptosis (30% decrease vs 70%; Figure 6). Additionally, the absolute level of basal apoptosis was greater in herstatin-expressing cells, and this was associated with increased cleavage of PARP (Figure 6).

Herstatin blocks EGF signaling

A number of studies have demonstrated that the EGFR is involved in IGF-I signaling (1, 5, 29-32). Therefore, the observed effects on IGF-I signaling may have been an indirect effect of herstatin-mediated inhibition of the EGFR. To determine whether EGF-

stimulated signaling was attenuated by herstatin, we compared the ability of EGF to activate the ERK and PI3K-Akt/PKB cascades in control and herstatin-expressing MCF-7 cells. As shown in Fig. 7, EGF treatment of control cells elicited robust ERK and Akt/PKB phosphorylation, which was severely reduced in cells expressing herstatin. These data demonstrate that herstatin blocks both heregulin and EGF-stimulated signaling in MCF-7 cells.

Effect of EGFR inhibition on IGF-IR expression

Herstatin expression had a striking effect on the levels of the IGF-IR. To determine if the observed effects of herstatin on IGF-IR levels were an indirect result of decreased EGFR action, we investigated whether specific inhibition of EGFR mimicked the effects of herstatin. Treatment with the EGFR inhibitor, AG1478, prevented EGF-stimulated activation of ERK (data not shown). However, neither short-term nor long-term treatment with AG1478 resulted in the down-regulation of IGF-IR levels that was seen in herstatin-expressing cells (Fig. 8).

DISCUSSION

An understanding of the effects of herstatin, an autoinhibitor of the EGFR family, on IGF-I signaling is critical to defining the overall mode of action of herstatin and to further clarify the mechanisms that link the actions of these two important RTK families. We have previously shown that herstatin blocks heregulin signaling and proliferation in MCF-7 cells (50). This study shows that EGF signaling is also blocked in these cells. To further assess the interplay between herstatin and the IGF-IR, initially suggested by binding of herstatin at nM concentrations to the ectodomain of the IGF-IR (47), we examined IGFI signaling and proliferation in MCF-7 breast carcinoma cells in which signaling through the EGFR family is disabled.

We found a striking effect of herstatin expression on several aspects of IGF-I signaling. Foremost, herstatin expression resulted in down-regulation of IGF-IR expression and an 8-fold decrease in IGF-I-induced IGF-IR tyrosine phosphorylation (Fig 1 and 2). Herstatin expression also resulted in a striking decrease in IRS-1 activation, which is immediately downstream of the IGF-IR in the IGF-I signaling pathway (Fig 3). Most importantly, this altered signaling was associated with inhibition of IGF-I inhibition of apoptosis in herstatin-expressing MCF-7 cells (Fig. 6).

In contrast to the blockade of EGF and heregulin-induced ERK activation, IGF-I stimulation of ERK was not inhibited, even though IGF-IR levels were reduced several fold (Fig. 4). Therefore, the extent of IGF-IR activation did not parallel the effects on the downstream ERK signaling cascade. Thus, the low levels of activated IGF-IR appeared to be sufficient to fully activate ERK signaling. Although ERK1 activation was unaffected, we observed a shift in the size of ERK1 in herstatin-expressing cells. We speculate that this size shift may be due to alternative splicing of the ERK1 gene, and may represent the ERK1b splice variant, which is 2.6 kDa larger than ERK1 (55-57). ERK1b has an altered ability to interact with MEK1 and may, therefore, result in a differential signaling profile (56). Interestingly, in herstatin-expressing cells, we also observed a preferential activation of ERK2 relative to ERK1 (Fig 3). Recent studies have implicated activation of ERK2, but not ERK1, in apoptosis (58-61). Therefore, the preferential activation of ERK2 in herstatin-expressing cells may contribute to the loss of IGFI-mediated inhibition of apoptosis demonstrated in Fig. 6.

The effects of herstatin expression on the signaling factors immediately downstream of the IGF-IR, IRS-1 and IRS-2, were complex and distinct. Herstatin reduced both IRS-1 expression and immunoprecipitation efficiency, with a concomitant decrease in IGF-I-stimulated tyrosine phosphorylation (Fig 1 and Figure 3 A & B). The mechanisms responsible for the two former effects are unclear. With respect to the differential immunoprecipitation of IRS-1 in control vs herstatin-expressing cells, it is possible that herstatin alters the subcellular localization or association pattern of IRS-1, such that the availability of IRS-1 to interact with multiple antibodies is attenuated. One possibility is that nuclear translocation of IRS-1, which has been observed in multiple cell types, including MCF-7 cells, is affected by herstatin expression (62). In contrast, herstatin expression did not significantly affect expression or activation of IRS-2. The differential enhancement of IGF-I-stimulated IRS-1 and IRS-2 activation by herstatin may reflect the fact that feedback mechanisms, such as patterns of inhibitory serine phosphorylation, differ between IRS-1 and IRS-2 (63). Interestingly, previous studies have shown that IRS-1, but not IRS-2, is important in IGF-I-mediated inhibition of apoptosis (64), an effect that may underlie the inhibitory effects of herstatin on cell viability seen in the current study. Combinatorial effects of herstatin expression that include decreased expression and activation of the IGF-IR and its immediate downstream signaling molecule, IRS-1, reduction in activation of Akt, and an increase in activation of ERK2, may all contribute to the retarded growth of herstatin-expressing MCF-7 cells (Fig. 5).

There are several potential mechanisms through which herstatin may modulate IGF-IR signal transduction and, thereby, IGF-I action. First, herstatin may directly bind to intracellular IGF-IR in the secretory pathway; alternatively, secreted herstatin may interact at the cell surface, since we have previously determined that it binds to the ectodomain of the IGF-IR with nanomolar affinity (48). However, since herstatin binds to all EGFR family members, and with higher affinity than to IGF-IR, the impact of herstatin on IGF-I signaling may be indirect and needs to be further investigated in cells that do not express the EGFR family.

A second possibility is that the modulation of IGF-I signaling is a secondary effect due to blockade of EGFR family signaling. Ample evidence exists for an IGF-I-stimulated autocrine loop that results in the release of heparin-binding EGF (HB-EGF) and, consequently, in the activation of the EGFR (32). To examine whether the effect of herstatin on down-regulation of the IGF-IR occurs via the EGFR, we blocked EGFR activation (using the EGFR-specific kinase inhibitor, AG1478) in parental MCF-7 cells. While the inhibitor fully blocked EGF-induced ERK activation (data not shown), it failed to mimic herstatin-mediated down-regulation of the IGF-IR (Fig. 8). However, we cannot rule out the possibility that longer-term effects of herstatin expression are involved, or that modulation of the other members of the EGFR family indirectly affects IGF-I signaling.

A third possibility is that herstatin may modulate the formation of hetero-oligomers between the IGF-I and EGF receptors. Recent evidence suggests that the EGFR is present in IGF-IR immunoprecipitates, suggesting the interesting possibility that herstatin may disrupt EGFR/IGF-IR hetero-oligomers (29). Regardless of whether this mechanism entails a direct or indirect effects of herstatin on the IGF-IR, the results presented here demonstrate a profound modulation of IGF-I signaling by an alternative product of the HER-2 gene.

The roles of both the EGFR and IGF-IR families in neoplastic growth and malignancies have been well documented. Over-expression and autocrine stimulation of both receptor families and their ligands has been implicated in a variety of carcinomas (65-69). Acquired resistance to Iressa, an EGFR inhibitor, in breast and prostate cancer cells is mediated by activation and signaling of the IGF-IR (37,38). Furthermore, IGF-IR signaling has been shown to protect HER-2-overexpressing breast carcinoma cells from the inhibitory effects of Herceptin, an anti-HER-2 monoclonal antibody (35). Thus, therapeutic strategies that are directed at both of these signaling systems would be

expected to have significant advantages over those that target a single growth factor pathway. Our data suggest that herstatin is an inhibitor that may block proliferative signals from two distinct families of RTKs.

The data obtained in this study were obtained with MCF-7 cells and were based on two independent herstatin-expressing clones in comparison to control cells. Although MCF-7 cells are a valuable and established model for the study of cellular regulatory mechanisms relevant to breast cancer, it will be desirable to extend these results to other cell types. Constitutive expression of herstatin is, however, toxic to most other cells that we have analyzed; thus, further studies will be facilitated by exploiting conditional, regulated expression models that we are currently developing.

Current receptor-directed therapeutics are typically targeted at a single receptor or receptor family, which may explain, in part, their limited clinical efficacy. Recently, a hetero-bi-functional monoclonal antibody that targets both the EGFR and IGF-IR was found to block both EGF and IGF-I-induced activation of Akt/PKB and ERK, resulting in strong inhibition of xenograft growth (45,46). We suggest that herstatin may have significant promise as a novel anti-cancer agent, since it acts as a multi-functional inhibitor that suppresses signaling from both the EGFR and IGF-IR families.

REFERENCES

1. Adams, T.E., McKern, N.M., and Ward, C.W. (2004) *Growth Factors* **22**(2), 89-95

2. Burden, S., and Yarden, Y. (1997) *Neuron* **18**(6), 847-855

3. Olayioye, M.A., Neve, R.M., Lane, H.A., and Hynes, N.E. (2000) *Embo J* **19**(13), 3159-3167

4. Sweeney, C., and Carraway, K.L., 3rd. (2000) *Oncogene* **19**(49), 5568-5573

5. Kuribayashi, A., Kataoka, K., Kurabayashi, T., and Miura, M. (2004) *Endocrinology* **145**(11), 4976-4984

6. Sell, C., Dumenil, G., Deveaud, C., Miura, M., Coppola, D., DeAngelis, T., Rubin, R., Efstratiadis, A., and Baserga, R. (1994) *Mol Cell Biol* **14**(6), 3604-3612

7. O'Connor, R., Kauffmann-Zeh, A., Liu, Y., Lehar, S., Evan, G.I., Baserga, R., and Blattler, W.A. (1997) *Mol Cell Biol* **17**(1), 427-435

8. Baserga, R. (1997) *Exp Cell Res* **236**(1), 1-3

9. Baserga, R., Resnicoff, M., D'Ambrosio, C., and Valentinis, B. (1997) *Vitam Horm* **53**, 65-98

10. Morrione, A., Romano, G., Navarro, M., Reiss, K., Valentinis, B., Dews, M., Eves, E., Rosner, M.R., and Baserga, R. (2000) *Cancer Res* **60**(8), 2263-2272

11. Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloen, A., Even, P.C., Cervera, P., and Le Bouc, Y. (2003) *Nature* **421**(6919), 182-187

12. Blume-Jensen, P., and Hunter, T. (2001) *Nature* **411**(6835), 355-365

13. Holbro, T., Civenni, G., and Hynes, N.E. (2003) *Exp Cell Res* **284**(1), 99-110

14. Pollak, M.N., Schernhammer, E.S., and Hankinson, S.E. (2004) *Nat Rev Cancer* **4**(7), 505-518

15. Rochester, M.A., Riedemann, J., Hellawell, G.O., Brewster, S.F., and Macaulay, V.M. (2005) *Cancer Gene Ther* **12**(1), 90-100

16. Hanks, S.K., Quinn, A.M., and Hunter, T. (1988) *Science* **241**(4861), 42-52

17. Schlessinger, J., Ullrich, A., Honegger, A. M., and Moolenaar, W.H. (1988) *Cold Spring Harb Symp Quant Biol* **53 Pt 1**, 515-519

18. Yarden, Y., and Ullrich, A. (1988) *Annu Rev Biochem* **57**, 443-478

19. Dougall, W.C., Qian, X., Peterson, N.C., Miller, M.J., Samanta, A., and Greene, M.I. (1994) *Oncogene* **9**(8), 2109-2123

20. Hynes, N.E. (2000) *Breast Cancer Res* **2**(3), 154-157

21. Riese, D.J., 2nd, and Stern, D.F. (1998) *Bioessays* **20**(1), 41-48

22. Tzahar, E., and Yarden, Y. (1998) *Biochim Biophys Acta* **1377**(1), M25-37
23. Rommel, C., Clarke, B. A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K., Moelling, K., Yancopoulos, G.D., and Glass, D.J. (1999) *Science* **286**(5445), 1738-1741
24. Busse, D., Doughty, R.S., Ramsey, T.T., Russell, W.E., Price, J.O., Flanagan, W.M., Shawver, L.K., and Arteaga, C.L. (2000) *J Biol Chem* **275**(10), 6987-6995
25. Ward, C.W., Garrett, T.P., McKern, N.M., Lou, M., Cosgrove, L.J., Sparrow, L.G., Frenkel, M.J., Hoyne, P.A., Elleman, T.C., Adams, T.E., Lovrecz, G.O., Lawrence, L.J., and Tulloch, P.A. (2001) *Mol Pathol* **54**(3), 125-132
26. Massague, J., and Czech, M.P. (1982) *J Biol Chem* **257**(9), 5038-5045
27. O'Connor, R. (2003) *Horm Metab Res* **35**(11-12), 771-777
28. Swantek, J.L., and Baserga, R. (1999) *Endocrinology* **140**(7), 3163-3169
29. Ahmad, T., Farnie, G., Bundred, N.J., and Anderson, N.G. (2004) *J Biol Chem* **279**(3), 1713-1719
30. Gilmore, A.P., Valentijn, A.J., Wang, P., Ranger, A.M., Bundred, N., O'Hare, M.J., Wakeling, A., Korsmeyer, S.J., and Streuli, C.H. (2002) *J Biol Chem* **277**(31), 27643-27650
31. El-Shewy, H.M., Kelly, F.L., Barki-Harrington, L., and Luttrell, L.M. (2004) *Mol Endocrinol* **18**(11), 2727-2739
32. Roudabush, F.L., Pierce, K.L., Maudsley, S., Khan, K.D., and Luttrell, L.M. (2000) *J Biol Chem* **275**(29), 22583-22589
33. Agus, D.B., Gordon, M.S., Taylor, C., Natale, R.B., Karlan, B., Mendelson, D.S., Press, M.F., Allison, D.E., Sliwkowski, M.X., Lieberman, G., Kelsey, S.M., and Fyfe, G. (2005) *J Clin Oncol*
34. Ross, J.S., Schenkein, D.P., Pietrusko, R., Rolfe, M., Linette, G.P., Stec, J., Stagliano, N.E., Ginsburg, G.S., Symmans, W.F., Pusztai, L., and Hortobagyi, G.N. (2004) *Am J Clin Pathol* **122**(4), 598-609
35. Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D., and Pollak, M. (2001) *J Natl Cancer Inst* **93**(24), 1852-1857
36. Chakravarti, A., Loeffler, J. S., and Dyson, N.J. (2002) *Cancer Res* **62**(1), 200-207
37. Camp, E. R., Summy, J., Bauer, T.W., Liu, W., Gallick, G.E., and Ellis, L.M. (2005) *Clin Cancer Res* **11**(1), 397-405
38. Jones, H.E., Goddard, L., Gee, J.M., Hiscox, S., Rubini, M., Barrow, D., Knowlden, J. M., Williams, S., Wakeling, A.E., and Nicholson, R.I. (2004) *Endocr Relat Cancer* **11**(4), 793-814

39. Garcia-Echeverria, C., Pearson, M.A., Marti, A., Meyer, T., Mestan, J., Zimmermann, J., Gao, J., Brueggen, J., Capraro, H.G., Cozens, R., Evans, D.B., Fabbro, D., Furet, P., Porta, D.G., Liebetanz, J., Martiny-Baron, G., Ruetz, S., and Hofmann, F. (2004) *Cancer Cell* **5**(3), 231-239
40. Mitsiades, C.S., Mitsiades, N.S., McMullan, C.J., Poulaki, V., Shringarpure, R., Akiyama, M., Hideshima, T., Chauhan, D., Joseph, M., Libermann, T.A., Garcia-Echeverria, C., Pearson, M.A., Hofmann, F., Anderson, K.C., and Kung, A.L. (2004) *Cancer Cell* **5**(3), 221-230
41. Burtrum, D., Zhu, Z., Lu, D., Anderson, D.M., Prewett, M., Pereira, D.S., Bassi, R., Abdullah, R., Hooper, A.T., Koo, H., Jimenez, X., Johnson, D., Apblett, R., Kussie, P., Bohlen, P., Witte, L., Hicklin, D.J., and Ludwig, D.L. (2003) *Cancer Res* **63**(24), 8912-8921
42. Maloney, E.K., McLaughlin, J.L., Dagdigian, N.E., Garrett, L.M., Connors, K.M., Zhou, X.M., Blattler, W.A., Chittenden, T., and Singh, R. (2003) *Cancer Res* **63**(16), 5073-5083
43. Sachdev, D., Li, S.L., Hartell, J.S., Fujita-Yamaguchi, Y., Miller, J.S., and Yee, D. (2003) *Cancer Res* **63**(3), 627-635
44. Camirand, A., Lu, Y., and Pollak, M. (2002) *Med Sci Monit* **8**(12), BR521-526
45. Lu, D., Zhang, H., Koo, H., Tonra, J., Balderes, P., Prewett, M., Corcoran, E., Mangalampalli, V., Bassi, R., Anselma, D., Patel, D., Kang, X., Ludwig, D.L., Hicklin, D. J., Bohlen, P., Witte, L., and Zhu, Z. (2005) *J Biol Chem*
46. Lu, D., Zhang, H., Ludwig, D., Persaud, A., Jimenez, X., Burtrum, D., Balderes, P., Liu, M., Bohlen, P., Witte, L., and Zhu, Z. (2004) *J Biol Chem* **279**(4), 2856-2865
47. Doherty, J.K., Bond, C., Jardim, A., Adelman, J.P., and Clinton, G.M. (1999) *Proc Natl Acad Sci U S A* **96**(19), 10869-10874
48. Shamieh, L.S., Evans, A.J., Denton, M.C., and Clinton, G.M. (2004) *FEBS Lett* **568**(1-3), 163-166
49. Justman, Q.A., and Clinton, G.M. (2002) *J Biol Chem* **277**(23), 20618-20624
50. Jhabvala-Romero, F., Evans, A., Guo, S., Denton, M., and Clinton, G.M. (2003) *Oncogene* **22**(50), 8178-8186
51. Azios, N.G., Romero, F.J., Denton, M.C., Doherty, J.K., and Clinton, G.M. (2001) *Oncogene* **20**(37), 5199-5209

52. Garrett, T.P., McKern, N.M., Lou, M., Elleman, T.C., Adams, T.E., Lovrecz, G.O., Zhu, H.J., Walker, F., Frenkel, M.J., Hoyne, P.A., Jorissen, R.N., Nice, E.C., Burgess, A.W., and Ward, C.W. (2002) *Cell* **110**(6), 763-773
53. Sambrook, J., Maniatis, T., and Fritsch, E.F. (1989) *Molecular cloning: a laboratory manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
54. Staverosky, J.A., Muldoon, L.L., Guo, S., Evans, A.J., Neuwelt, E.A., and Clinton, G.M. (2005) *Clin Cancer Res* **11**(1), 335-340
55. Aebersold, D.M., Shaul, Y.D., Yung, Y., Yarom, N., Yao, Z., Hanoch, T., and Seger, R. (2004) *Mol Cell Biol* **24**(22), 10000-10015
56. Yung, Y., Yao, Z., Aebersold, D.M., Hanoch, T., and Seger, R. (2001) *J Biol Chem* **276**(38), 35280-35289
57. Yung, Y., Yao, Z., Hanoch, T., and Seger, R. (2000) *J Biol Chem* **275**(21), 15799-15808
58. Castro-Obregon, S., Rao, R.V., del Rio, G., Chen, S.F., Poksay, K.S., Rabizadeh, S., Vesce, S., Zhang, X.K., Swanson, R.A., and Bredesen, D.E. (2004) *J Biol Chem* **279**(17), 17543-17553
59. Chen, J.R., Plotkin, L.I., Aguirre, J.I., Han, L., Jilka, R. L., Kousteni, S., Bellido, T., and Manolagas, S.C. (2005) *J Biol Chem* **280**(6), 4632-4638
60. Cheung, E. C., and Slack, R.S. (2004) *Sci STKE* **2004**(251), PE45
61. Frese, S., Pirnia, F., Miescher, D., Krajewski, S., Borner, M.M., Reed, J.C., and Schmid, R.A. (2003) *Oncogene* **22**(35), 5427-5435
62. Morelli, C., Garofalo, C., Sisci, D., del Rincon, S., Cascio, S., Tu, X., Vecchione, A., Sauter, E.R., Miller, W.H., Jr., and Surmacz, E. (2004) *Oncogene* **23**(45), 7517-7526
63. Kim, J.A., Yeh, D.C., Ver, M., Li, Y., Carranza, A., Conrads, T.P., Veenstra, T.D., Harrington, M.A., and Quon, M.J. (2005) *J Biol Chem* **280**(24), 23173-23183
64. Tseng, Y.H., Ueki, K., Kriauciunas, K.M., and Kahn, C.R. (2002) *J Biol Chem* **277**(35), 31601-31611
65. Harari, P. M. (2004) *Endocr Relat Cancer* **11**(4), 689-708
66. Khandwala, H.M., McCutcheon, I.E., Flyvbjerg, A., and Friend, K.E. (2000) *Endocr Rev* **21**(3), 215-244
67. Mendelsohn, J. (2001) *Endocr Relat Cancer* **8**(1), 3-9
68. Slamon, D.J., and Clark, G.M. (1988) *Science* **240**(4860), 1795-1798
69. Yarden, Y., and Sliwkowski, M.X. (2001) *Nat Rev Mol Cell Biol* **2**(2), 127-137

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Effect of herstatin expression on the expression levels of IGF signaling proteins. Sub-confluent MCF-7 and MCF-7/Hst cells were extracted and signaling protein levels were assessed by Western blot.

Figure 2. Herstatin modulation of IGF-I activation of the IGF-IR. MCF-7 and MCF-7/Hst cells were serum-starved overnight, treated with 5 nM IGF-I over a 60-minute time course, and harvested in NP-40 lysis buffer. 1 mg of cell lysate was immunoprecipiated with an IGF-IR antibody and protein A-agarose beads. Immunoprecipitates were separated on a 10% SDS-PAGE gel and analyzed for IGF-IR expression and tyrosine phosphorylation using anti-IGF-IR and PY20 anti-phosphotyrosine antibodies, respectively. Western blots were scanned and quantified by densitometry. **(A)** Representative Western blot of IGF-IR immunoprecipitated from IGF-I-treated MCF-7 and MCF-7/Hst cells. **(B)** A graphical representation of two independent experiments of IGF-I-induced activation of the IGF-I receptor.

Figure 3. Effect of herstatin on IGF-I activation of IRS-1 and IRS-2. MCF-7 and MCF-7/Hst cells were serum-starved overnight, treated with 5 nM IGF-I over a 60-minute time course, and harvested in NP-40 lysis buffer. 1 mg of cell lysate was immunoprecipitated with IRS-1 (A & B) or IRS-2 (C & D) antibodies and protein A-agarose beads. Immunoprecipitates were separated on a 10% SDS-PAGE gel and analyzed for IRS expression and tyrosine phosphorylation. Western blots were scanned and quantified by densitometry. **(A)** Representative IRS-1 immunoprecipitation and analysis with antiphosphotyrosine PY20 antibody. Both light and dark exposures of the IRS-1 immunoprecipitation are shown. **(B)** Graphical representation of 3 separate experiments. **(C)** Representative IRS-2 immunoprecipitation and analysis with anti-phosphotyrosine PY20 antibody. **(D)** Graphical representation of 3 separate experiments.

Figure 4. Effect of herstatin on IGF-I activation of ERK. MCF-7 and MCF-7/Hst cells were serum-starved and treated with 5 nM IGF-I at 37ºC over a 60-minute time course. Cell lysates (50 μg) were separated on a 10% SDS-PAGE gel and then analyzed by Western blotting with ERK and phospho-ERK antibodies. **(A)** Representative Western

blot showing IGF-I-induced ERK activation in MCF-7 and MCF-7/Hst cells. **(B)** Graphical representation of 3 separate experiments.

Figure 5. Effect of herstatin on IGF-I activation of Akt/PKB. MCF-7 and MCF-7/Hst cells were serum-starved and treated with 5nM IGF-I at 37ºC over a 60- minute time course. Cell lysates (50 µg) were separated on a 10% SDS-PAGE gel and then analyzed by Western blotting with Akt and phospho-Akt antibodies. Western blots were scanned and quantified by densitometry. **(A)** Representative Western blot showing IGF-I-induced Akt/PKB activation in MCF-7 and MCF-7/Hst cells. **(B)** Graphical representation of 3 separate experiments.

Figure 6. Effect of herstatin on basal and IGF-I-inhibited apoptosis. MCF-7 and MCF-7/Hst cells were serum-starved for 24 hours and then treated with 5 nM IGF-I or vehicle. Apoptosis was determined by a cell death ELISA assay as described in Materials and Methods and was assessed after 48 hrs (upper panel). PARP cleavage was assessed by western immunoblotting (lower panel).

Figure 7. Effect of herstatin on EGF-stimulated signaling. MCF-7 and MCF-7/Hst cells were serum-starved and treated with 5 nM EGF at 37ºC for the times indicated. Cells were lysed, and lysates were run on a 10% SDS-PAGE gel and ERK and Akt activation were analyzed by Western blotting as described in the legends to Figures 3 and 4. Western blots were scanned and quantified by densitometry. **(A)** Effect of herstatin expression on EGF-induced ERK activation. **(B)** Effect of herstatin expression on EGF-induced Akt/PKB activation.

Figure 8. Effect of AG1478 on IGF-IR expression. MCF-7 cells were treated with AG1478 for the times indicated. Cells were lysed and lysates were run on a 10% SDS-PAGE gel and analyzed by Western blot.

Figure 1.

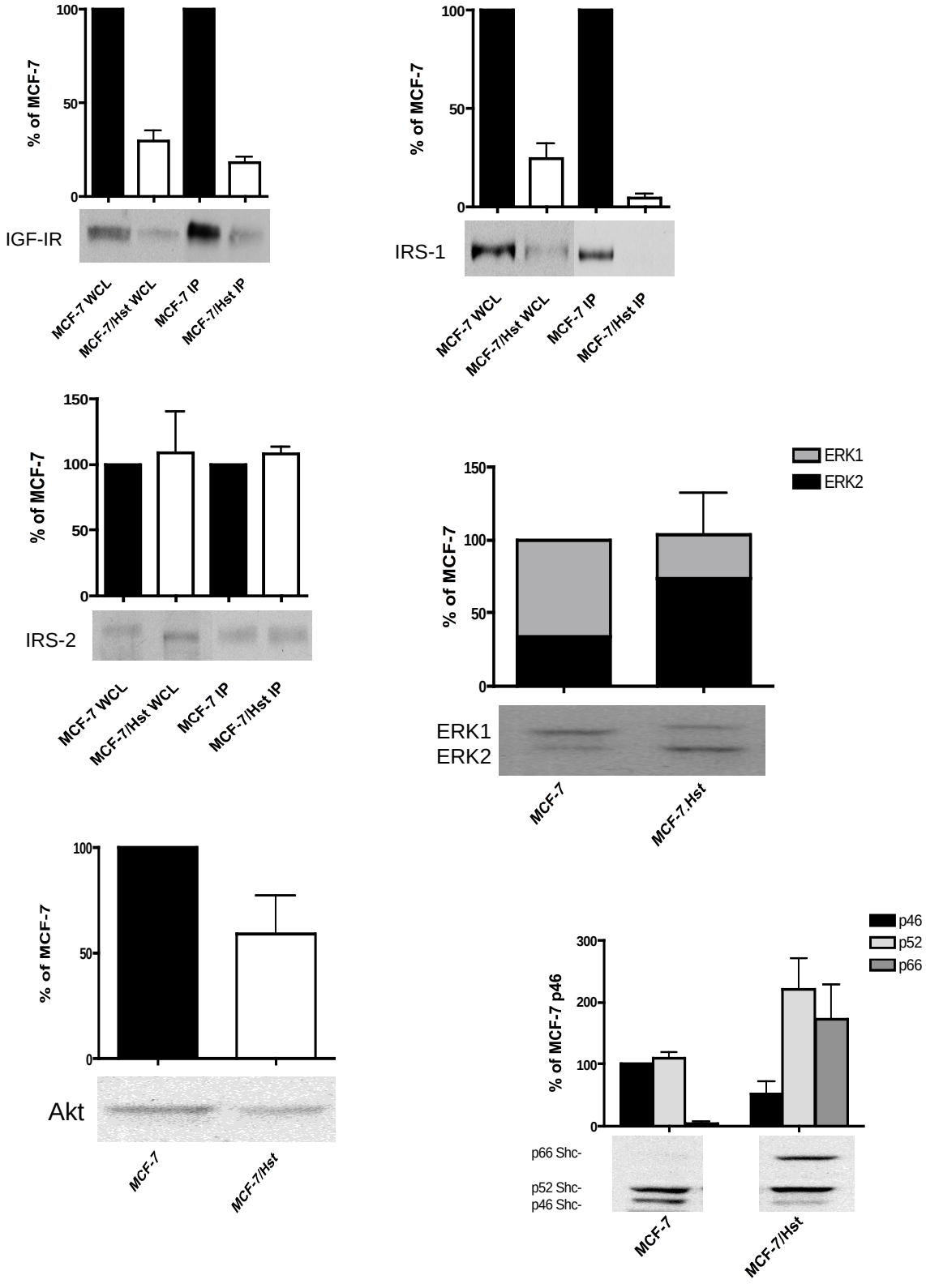

% of MCF-7
100
50
0
IGF-IR
MCF-7 WCL
MCF-7/Hst WCL
MCF-7 IP
MCF-7/Hst IP
IRS-1
IRS-2
ERK1
ERK2
MCF-7
MCF-7.Hst
Akt
% of MCF-7 p46
300
200
p46
p52
p66
p66 Shc-
p52 Shc-
p46 Shc-

Figure 2.

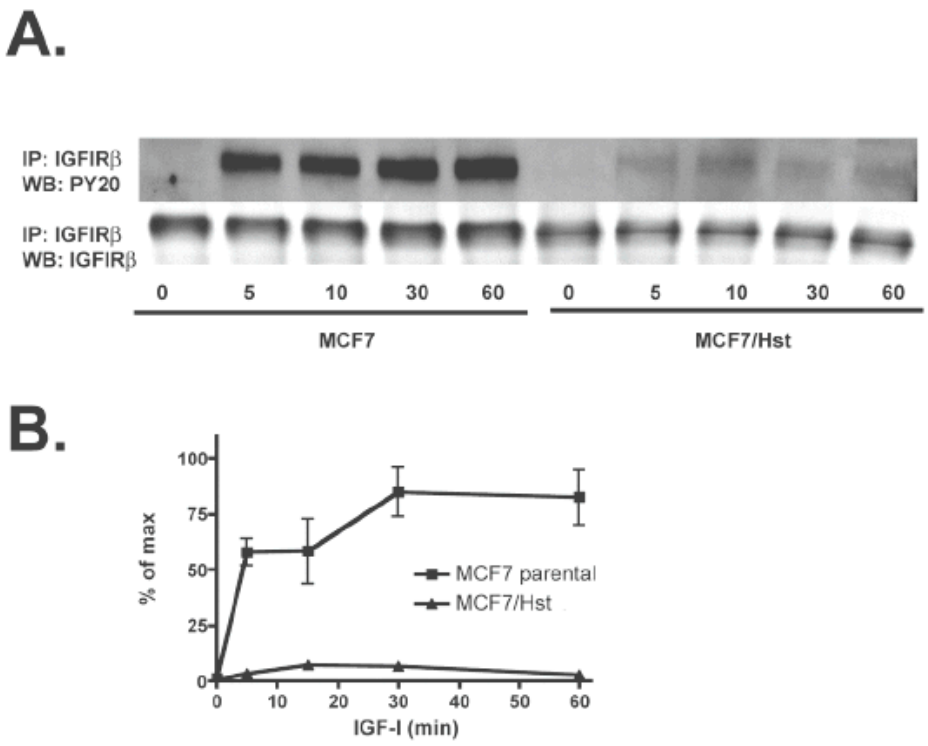

A.
IP: IGFIRβ
WB: PY20
IP: IGFIRβ
WB: IGFIRβ
0
5
10
30
60
0
5
10
30
60
MCF7
MCF7/Hst
B.
% of max
100
75
50
25
0
MCF7 parental
MCF7/Hst
0
10
20
30
40
50
60
IGF-I (min)

Figure 3.

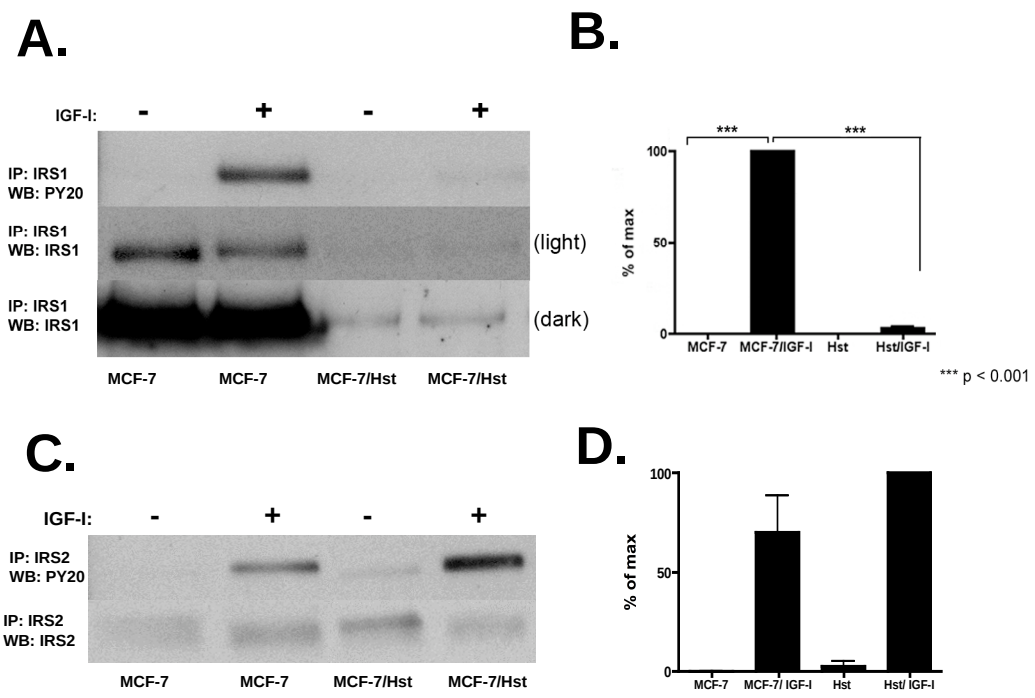

A.
IGF-I: - + - +
IP: IRS1
WB: PY20
IP: IRS1
WB: IRS1
(light)
IP: IRS1
WB: IRS1
(dark)
MCF-7
MCF-7
MCF-7/Hst
MCF-7/Hst
B.
***
***
100
50
0
% of max
MCF-7
MCF-7/IGF-I
Hst
Hst/IGF-I
*** p < 0.001
C.
IGF-I: - + - +
IP: IRS2
WB: PY20
IP: IRS2
WB: IRS2
MCF-7
MCF-7
MCF-7/Hst
MCF-7/Hst
D.
100
50
0
% of max
MCF-7
MCF-7/ IGF-I
Hst
Hst/ IGF-I

Figure 4.

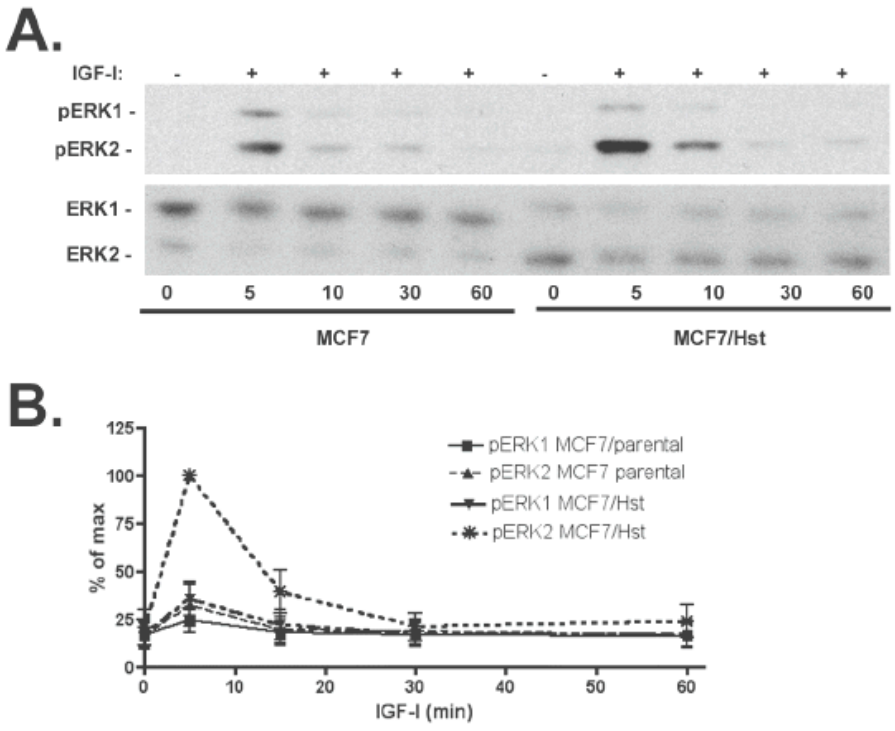

A.
IGF-I: - + + + + - + + + +
pERK1 -
pERK2 -
ERK1 -
ERK2 -
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst
B.
pERK1 MCF7/parental
pERK2 MCF7 parental
pERK1 MCF7/Hst
pERK2 MCF7/Hst
% of max
125
100
75
50
25
0
0 10 20 30 40 50 60
IGF-I (min)

Figure 5.

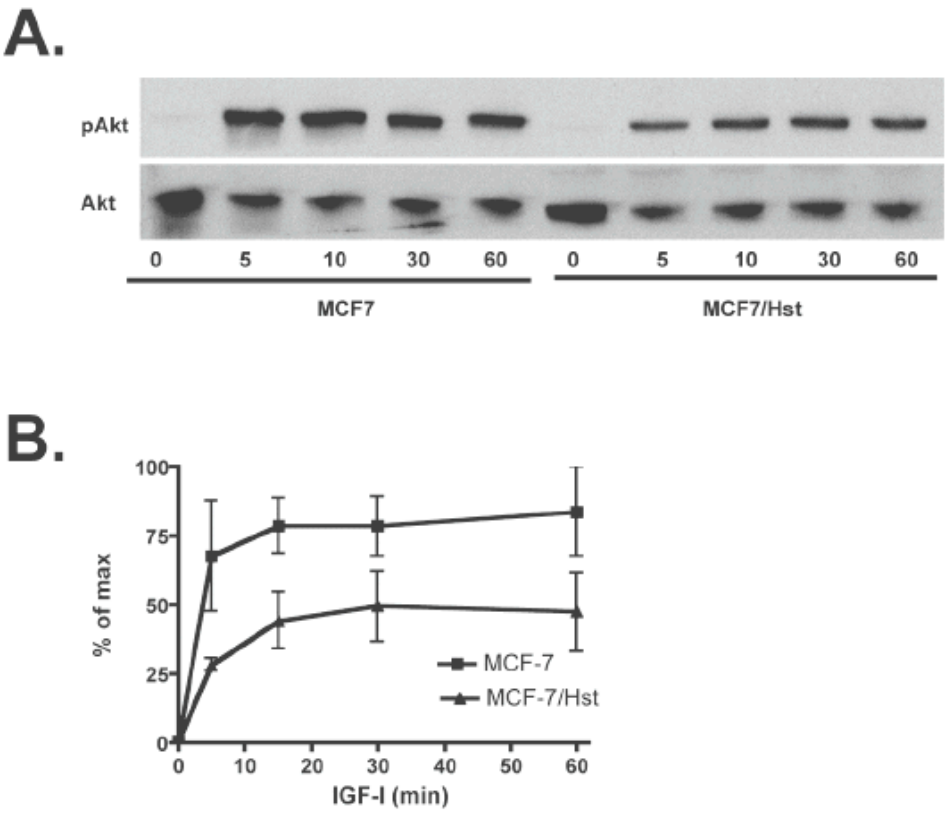

A.
pAkt
Akt
0
5
10
30
60
0
5
10
30
60
MCF7
MCF7/Hst
B.
100
75
50
25
0
% of max
MCF-7
MCF-7/Hst
0
10
20
30
40
50
60
IGF-I (min)

Figure 6.

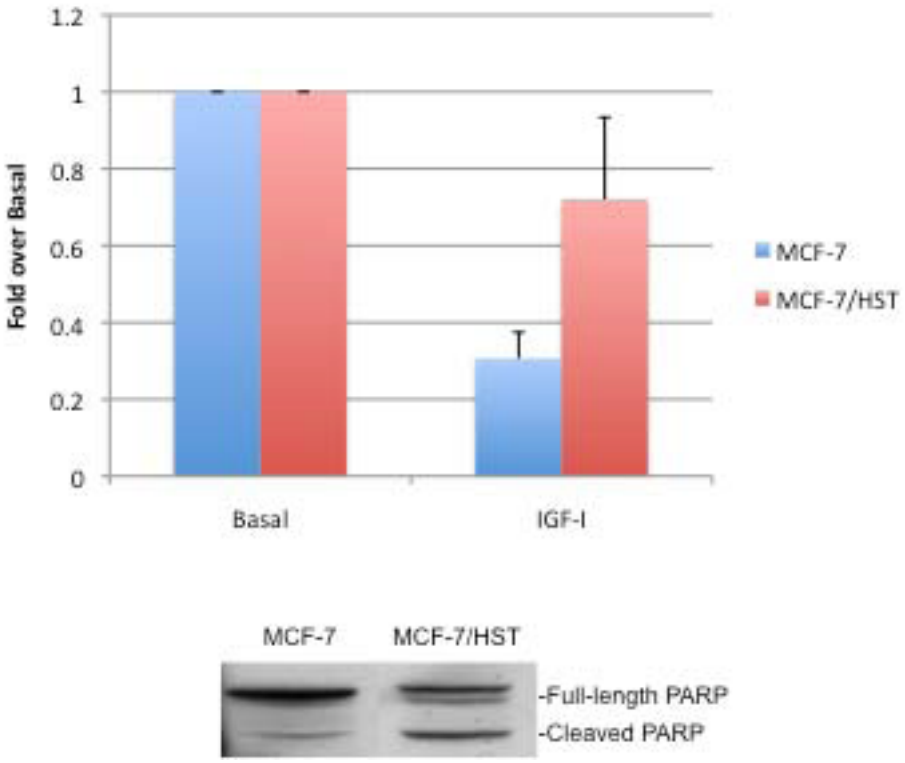

1.2
1
0.8
0.6
0.4
0.2
0
Fold over Basal
Basal
IGF-I
MCF-7
MCF-7/HST
MCF-7
MCF-7/HST
-Full-length PARP
-Cleaved PARP

Figure 7.

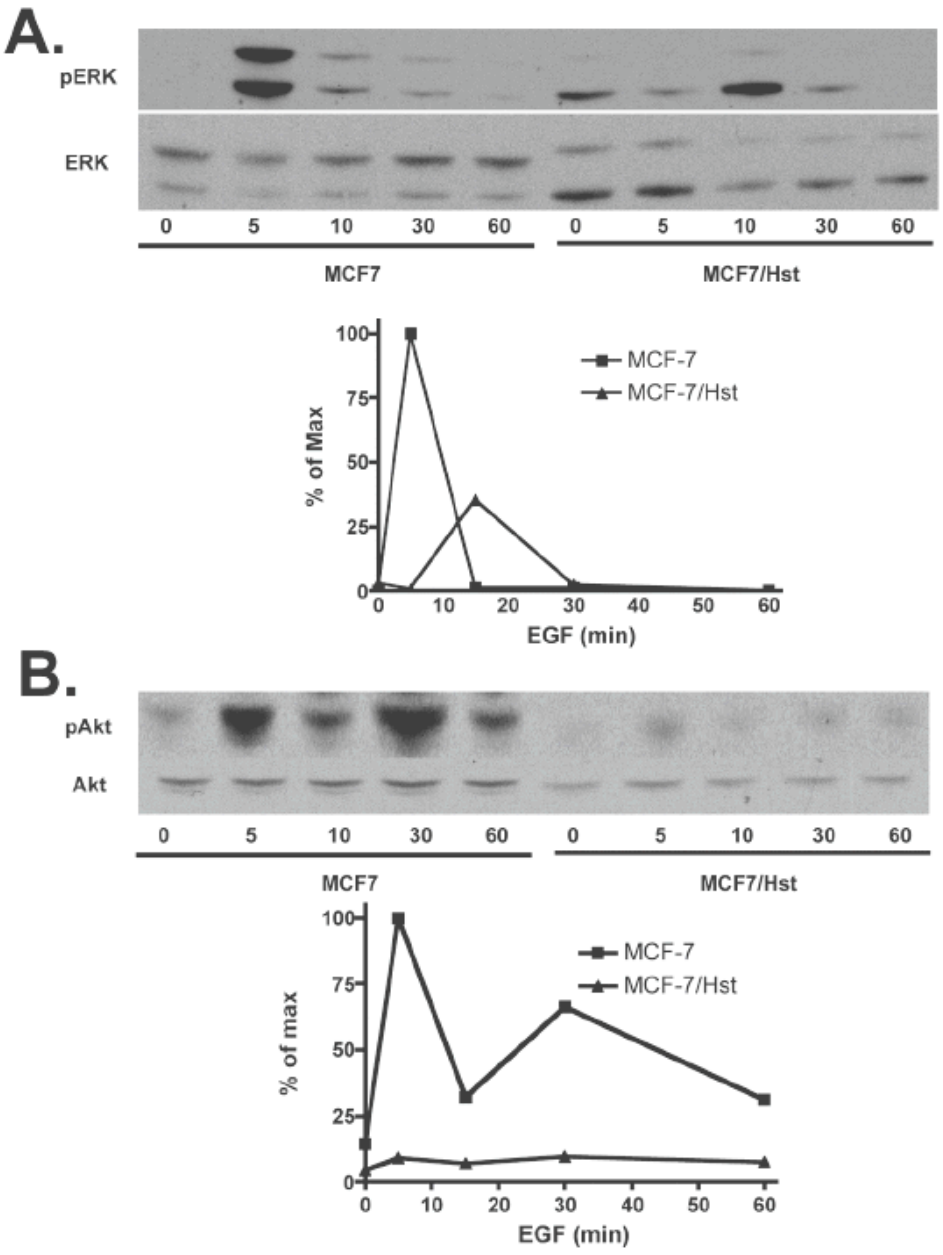
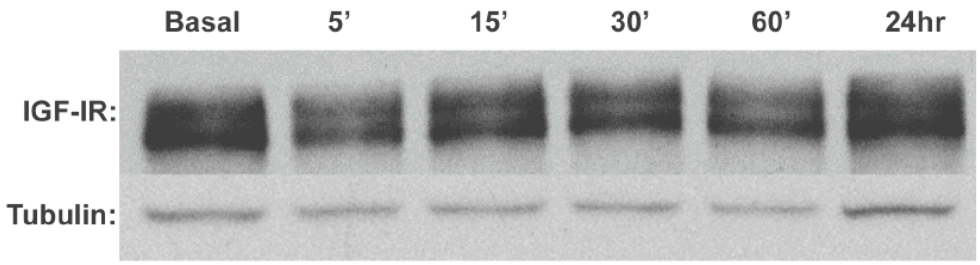

A.
pERK
ERK
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst
100
75
50
25
0
% of Max
MCF-7
MCF-7/Hst
0 10 20 30 40 50 60
EGF (min)
B.
pAkt
Akt
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst
100
75
50
25
0
% of max
MCF-7
MCF-7/Hst
0 10 20 30 40 50 60
EGF (min)

Figure 8.



OREGON HEALTH & SCIENCE UNIVERSITY

BIOSCIENCE INNOVATION PROGRAM

Grant Application

For questions please contact the OHSU Office of Technology & Research Collaborations at (503) 494-8200.

Title of Proposed Research Project:
Herstatin; a novel cancer therapeutic

Principal Investigator Name:
Charles T. Roberts, Jr., Ph.D.
Position and Title:
Associate Director, ONPRC, and Professor, Depts. of Medicine and Cell and Developmental Biology
Email:
robertsc@ohsu.edu
Telephone:
503-690-5259
Address:
L-584, 505 NW 185th Ave., Beaverton, OR 97006

OHSU Department and Unit:
Oregon National Primate Research Center
TRC Associate:
Arun Pradhan
Amount Requested: $80,000 for year 1

Term of Project:
Start Date: 3/1/2009 **End Date:** 2/28/2010

IRB or other Reviews or Certifications needed:
None

Agreement:

By submitting this application, the applicant agrees that:

1) funds awarded will be expended for the purposes of this project only;
2) any funds remaining at completion of the project will be returned to OHSU/OHSU Foundation;
3) the award may be revoked in whole or in part at any time at the discretion of the Vice-President for Research and Office of Technology and Research Collaborations for failure to meet the objectives specified in the proposal;
4) any revocation shall not include funds obligated prior to the date of revocation provided that such obligations were made solely for the purposes of this project;
5) the research performed under this award will be consistent with all university guidelines and policies; and
6) in the event that research supported by the award results in commercialization revenue to OHSU, the amount of the award will be deducted from such revenue prior to any distributions to the University or Inventors.

The information in this application is true and complete to the best of the applicant's knowledge.

_____ *12/21/2008*

Signature of Principal Investigator Date

I. Background

a. One page or less

Breast and prostate cancer represent the second and first most common types of non-skin cancers in women and men, respectively, and result in over 50,000 deaths annually in the US. Current therapies for breast cancer, in particular, target the estrogen receptor and the EGFR family of proteins that are found on the surface of cancer cells. The EGFR family includes the EGFR, HER2, HER3, and HER4 receptor tyrosine kinases, and is also implicated in prostate and lung cancer. The HER2 gene in particular is amplified in certain luminal breast cancers, and the EGFR is over-active in the particularly malignant basal-like category of breast cancer. EGFR and HER2 are the targets of the monoclonal antibodies Herceptin and the small-molecule tyrosine kinase inhibitors (TKIs) Iressa and Tarceva, all of which are in clinical use. The efficacy of these treatments is modest, however, in that they only extend survival by a few months, and only in conjunction with chemotherapy. This is because they inhibit EGFR and HER2, but not HER3, which is responsible for the tumorigenic effects of EGFR and HER2 through the formation of EGFR/HER3 and HER2/HER3 complexes in tumor cells. HER3 itself is not amenable to TKI drugs since it lacks intrinsic kinase activity. In other words, these drugs do not attack a wide enough range of targets to be sufficiently effective.

Herstatin is an alternative product of the HER2 gene, originally described Dr. Gail Clinton of OHSU in 1999. Herstatin is produced by the retention of intron 8 of the HER2 gene. As a result, a secreted protein comprised of the N-terminal portion of the HER2 extracellular domain (ECD) and a unique intron 8-specified C-terminal domain is generated. Previous work has shown that this intron-encoded domain is able to bind to all of the members of the EGFR family. Expression of herstatin in MCF-7 human breast cancer cells reduces EGFR, HER3, and HER4 expression and EGF ligand-stimulated cell growth and survival. More importantly, exposure of breast cancer cells to exogenous recombinant herstatin also reduces expression of EGFR family members and inhibits growth in vitro. The utility of herstatin as a novel cancer therapy is most directly demonstrated by data showing that injection of recombinant herstatin into mice inhibits the growth of xenografts of human prostate, ovarian, and stomach cancer cells.

Subsequent work in collaboration with Dr. Roberts demonstrated that herstatin also binds to and inhibits the expression of the IGF-I receptor (IGF-IR), and prevents IGF-stimulated IGF-IR activation and action. The significance of these findings is that the IGF-I system has also been implicated in numerous human cancers, including breast, lung, prostate, and colon, and there is substantial evidence for crosstalk between the IGF-IR and both EGFR and HER2 at the molecular level. Specifically, activation of the IGF-IR contributes to resistance to EGFR/HER2-directed TKIs in lung cancer, while, conversely, EGFR or HER2 activation induces resistance to an IGF-IR TKI in breast cancer cells. These data support the idea that inhibition of both the EGFR/HER and IGF-IR systems is desirable. Herstatin, therefore, represents a novel, bi-functional inhibitor of the EGFR/HER and IGF-IR pathways that, individually and collectively, are thought to drive the development and progression of multiple human cancers.

The advantages of herstatin as an alternative to current therapies are that: 1) herstatin is a naturally occurring protein that probably serves as an endogenous inhibitor of growth factor action; 2) by inhibiting EGFR, HER2, and HER3, it may exhibit greater efficacy than the TKIs now employed, principally through it's ability to inhibit HER3 as well as EGFR and HER2; and 3) by co-targeting both the EGFR/HER and IGF signaling systems, it represents a single-agent mechanism that may be more effective than combinations of antibodies and TKIs that target these systems separately. It is, of course, possible that herstatin may enhance the utility of existing EGFR/HER and IGF-IR-specific drugs.

BSIF funding will enable the performance of the necessary pre-clinical studies that will establish the utility of herstatin as a new cancer therapy, and will allow its development to the point at which it will be attractive to licensees or investors.

II. Description of Proposed Research

a. One page or less

The overall goal of this effort is to optimize the synthesis of herstatin or a smaller version and to validate efficacy in an accepted preclinical model (i.e., inhibition of tumorigenecity in xenograft and/or innate cancer models in mice. This data will serve as the impetus for outside investment that will enable production of lead compounds for clinical trials. Previous work at OHSU and at Receptor Biologix employed full-length herstatin protein produced in an insect cell system. This approach was hampered by the aggregation of herstatin, which necessitates the purification of the small proportion of monomeric material. An additional issue is the extent of appropriate post-translational modification that was achieved in this heterologous system, which could have influenced biological activity. A separate concern is the lack of understanding of the potential efficacy of smaller versions of herstatin that would be simpler to produce or which may be amenable to mimicking by small molecule/chemical biology approaches.

Our proposed initial studies will address these issues by generating recombinant herstatin in mammalian cells as well as synthesizing protein fragments of herstatin corresponding to the intron 8-encoded domain. The efficacy of the recombinant material will be assessed in vitro using transwell co-culture of herstatin-producing cells and a collection of cancer cell lines; the latter will be assessed for inhibition of EGFR family and IGF-IR action as well as cellular growth and resistance to programmed cell death. The activity of intron 8 domain synthetic peptides will be assessed similarly. If the smaller peptides are not sufficiently active, we will produce and analyze recombinant herstatin fragments comprised of the intron 8 domain and progressively larger portions of the HER2 ECD component in order to obtain the smallest version of herstatin that exhibits the desired activity in vitro. These studies will be completed in the first year. The specific experimental approaches are detailed below:

1. Generate lentivirus vectors containing a TPA signal sequence-herstatin-Sumo-FLAG fusion cassette and infect EGFRv1-expressing cos7 cells. The TPA signal sequence will result in efficient secretion, the Sumo fusion will facilitate correct folding of herstatin and is removable using Sumo protease, the EGFRv1-expressing cells will be resistant to the herstatin produced (this mutant EGFR can drive cos7 cell proliferation for protein production, but is not inhibited by herstatin), and the FLAG tag will facilitate detection and purification from conditioned media.
2. Herstatin-expressing cells will be co-cultured with various human cancer cell lines to assess effects on growth and other parameters.
3. Recombinant herstatin will be isolated from media, processed and tested on cancer cell lines to verify activity of the purified material.
4. Synthetic, 79-amino acid intron 8 protein and smaller fragments will be purchased and tested for inhibitory activity in cancer cell lines in vitro.
5. If necessary, lentivirus cassettes will be designed that encode fusions of fragments of the herstatin N and C-terminal domains to be tested as in approaches 1 and 2. Approaches 4 and 5 will be informed by molecular modeling in collaboration with our OHSU colleague Dr. Thomas Scanlan.

Our anticipated goals for year 2 will be to produce lead versions of herstatin in sufficient quantity to be employed in mouse xenograft studies or in innate mouse cancer models. These studies will be done in collaboration with Dr. Roberts' colleague at the University of Washington, Dr. Stephen Plymate, who is expert in mouse tumorigenesis studies. Simultaneously, additional

modification or derivitization of lead versions will be performed and in vitro evaluation done prior to assessment in vivo. Potential modeling of small-molecule analogs of suitably sized, biologically active herstatin fragments will be done in collaboration with our OHSU colleague Dr. Ujwal Shinde.

b. Summarize with research hurdles and result needed to clear the hurdle

Year-1 goals include the generation and analysis of native herstatin using a mammalian expression system and the assessment of activity of herstatin fragments as an alternative to full-length herstatin.

Hurdle	*Positive outcome*
Lentiviral vector design	Production of herstatin in infected cells
Purification of recombinant protein	Recovery of herstatin from culture media
Demonstration of biological activity	Inhibition of EGFR/HER and IGF-IR-stimulated growth of cancer cells in co-culture or with purified protein
Activity of intron 8-encoded domain	Above activity seen with 79-amino acid intron 8 peptide or smaller versions
Activity of herstatin fragments	Design, expression, and activity assessment of combinations of intron 8 domain with portions of HER2 ECD

III. Commercialization Milestones

a. One page or less

b. Commercial potential

Based upon its probable targets, the likely market for herstatin is a proportion of patients with breast, prostate, lung, and potentially other cancers, such as glioma, head and neck, endometrial, and ovarian. The current providers include Genentech (Herceptin and Tarceva), Astra-Zeneca (Iressa), GlaxoSmithKline (Lapatinib), and Imclone (Erbitux), whose collection of monoclonal antibody and TKI treatments produced approached $6 billion in sales in 2007. It is likely that the focus of R&D will remain on these classes of drugs, which will continue to be most effective in combination with chemotherapy.

c. Commercialization milestones

1. Initiate research supported by BSIF funding
2. Incorporate commercial entity with Springboard funding as OHSU start-up
3. Submit NIH SBIR applications based on current data
4. Solicit VC/private equity interest/investment based upon current and new data
5. Utilize VC/private equity investment for optimized lead production, IND filing, and preclinical toxicity, PD, and PK studies in non-human primates prior to phase-I trials

IV. Intellectual Property Position

V. Budget

a. Less than one page

Personnel-

Principal investigator (Dr. Charles Roberts @ 5% effort) $11,800 (to be obtained separately)

Senior Research Associate (Ms. Julie Carroll, M.S. @ 50% effort)		$30,375
Research Assistant 2 (Ms. Aubrey Ng, B.S. @ 50% effort)		$22,500
Supplies-		
Cell culture materials (plasticware, media)		$5,000
Biochemical reagents (antibodies, ECL reagents, protein gels)	$6,000	
Peptide synthesis	$10,000	
Lentivirus production		$2,500
Human cancer cell lines and EGFR vectors		$2,000
Apoptosis and proliferation assay kits	$1,625	
Total	$80,000	

Justification-

Personnel:

Dr. Charles Roberts will be responsible for overall project direction, data analysis, publications, and reporting on milestones/progress.

Ms. Julie Carroll is a senior technician who has extensive experience in growth factor action in cancer cells, and performed many of the studies on herstatin that are the basis for the current proposal. She will be responsible for the cellular signaling and other assays of cell growth, survival, and tumorigenesis.

Ms. Aubrey Ng is a research assistant with experience in molecular biology and biochemistry and will be responsible for vector construction and will assist Ms. Carroll in cell biology studies.

Supplies:

The supplies budgeted for are all consumables necessary for the studies proposed, and the amounts requested are best estimates based upon technically similar studies we have undertaken in the past.

Plasticware includes transwell inserts for assessment of action of secreted herstatin. Peptide synthesis will involve production of the intron 8-encoded protein and smaller fragments by a commercial vendor. Lentivurus constructs will be generated that encode full-length herstatin as well as combinations of the intron-encoded domain and portions of the HER2 ECD. Human cancer cell lines will be procured from ATCC to complement ones we have on hand.

VI. Attachments & References

a. Please provide any scientific or commercialization papers or references that may be helpful in supporting your application

Representative Pertinent References:

Herstatin inhibition of HER3-
Azios et al., Expression of herstatin, an autoinhibitor of HER-2/neu, inhibits transactivation of HER-3 by HER-2 and blocks EGF activation of the EGF receptor. Oncogene 20:5199-5209, 2001.

Molecular modeling of the herstatin-HER2 interaction-
Hu et al. In vivo identification of the interaction site of ErbB2 extracellular domain with its autoinhibitor. J. Cell. Physiol. 205:335-343, 2005.

Herstatin interaction with the IGF-IR-
Shamieh et al. Receptor binding specificities of herstatin and its intron 8-encoded domain. FEBS Lett. 568:163-166, 2004.

Role of HER3 in EGFR/HER-mediated tumorigenesis-
Hsieh and Moasser. Targeting HER proteins in cancer therapy and the role of the non-target HER3. Brit. J. Can. 97:453-457, 2007.
Sergina et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature 445:437-441, 2007.

IGF-IR-mediated resistance to EGFR/HER-targeted therapy-
Morgillo et al. Implication of the insulin-like growth factor-IR pathway in the resistance of non-small cell lung cancer cells to treatment with gefitinib. Clin. Can. Res. 13:2795-2803, 2007.

EGFR/HER2-mediated resistance to IGF-IR-targeted therapy-
Haluska et al. HER receptor signaling confers resistance to the insulin-like growth factor-I receptor inhibitor, BMS-536924. Mol. Can. Ther. 7:2589-2598, 2008.

Please Return the Completed Application to:

Technology & Research Collaborations
Mailcode: AD110
2525 SW 1st Avenue, Suite 110
Portland, OR 97201